Oxygen-Dependent Regulation of Base Excision Repair and its Role in Oxidative Stress Tolerance and Tumor Therapy Resistance

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To my family,
Vera, Maria, Fedya and Viktor,
for all their endless love, support and belief.

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ABBREVIATIONS

In alphabetical order

8-oxo-G	7,8-dihydro-8-oxoguanine
A	Adenine base
A	Alanine amino acid
AP site	Apurinic/apyrimidinic site, abasic site
APE1	Apurinic/apyrimidinic endonuclease 1
BER	Base excision repair
C	Cytosine base
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic acid
FEN1	Flap endonuclease 1
FIH	Factor inhibiting HIF
G	Guanine base
Gy	Gray, unit of radioactivity
H_2O_2	Hydrogen peroxide, an oxidative agent
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive element
HR	Homologous recombination
IR	Ionizing radiation
MEF	Mouse embryonic fibroblast
MMS	Methyl methanesulfonate, an alkylating agent
MUTYH	MutYH DNA glycosylase
NHEJ	Non-homologous end-joining
OGG1	OGG1 DNA glycosylase
PKB (Akt)	Protein kinase B, Akt
Pol	DNA polymerase
PTM	Posttranslational modification
pVHL	Von Hippel-Lindau protein
ROS	Reactive oxygen species
shRNA	Short-hairpin RNA
siRNA	Short-interfering RNA
S	Serine amino acid
T	Thymine base
vEGF	Vascular endothelial growth factor
WCE	Whole cell extract

SUMMARY

ENGLISH

Adaptation to a world with 20% oxygen in its atmosphere is closely linked to the ability of organisms to couple with oxygen's harmful potential. DNA damage by reactive oxygen species (ROS) is a major issue, since insufficient elimination of damaged bases (lesions) from DNA is deleterious and leads to cancer. Repair of DNA lesions including the most mutagenic 8-oxo-guanine (8-oxo-G) relies on base excision repair (BER) - a complex orchestra of 30 different proteins-, which are required to be subtly regulated in order to keep DNA repair activate only when necessary, thus preventing genomic instability. In the past years mechanisms regulating BER dependent on the cell cycle or in response to severe DNA damage rapidly induced by oxidizing agents and irradiation have been uncovered. However, how BER is regulated when DNA is continuously damaged through extensive exposure to ROS, generated by excessive oxygen consumption (like observed during tissue growth and regeneration), or contrary, in deprivation of oxygen (like tumor hypoxia), has not been clearly defined so far. Moreover, mechanisms controlling BER in these responses and their role in cell physiology and/or tumorgenesis have not been elucidated.

In this thesis work, the expression of four core BER genes – the two DNA glycosylases hOGG1 and MUTYH and the two DNA polymerases λ and β – was activated by hypoxia, and, in spite of gradual decline, was maintained, during the entire period of re-oxygenation, thus resulting in increased capacity of hOGG1 initiated BER, both under hypoxia and upon re-oxygenation. The capacity of hOGG1 mediated BER was controlled via the HIF-1α pathway, that under hypoxia induced both mRNA and protein levels of hOGG1 and DNA polymerase β. However, during re-oxygenation, the activity of the hOGG1 protein was also influenced by protein kinase B (PKB, Akt) dependent phosphorylation. Phosphorylation of the hOGG1 on Ser51 by Akt2 increased activity of hOGG1 mediated BER upon re-oxygenation, due to accelerated nuclear translocation. Simultaneously, the capacity of MUTYH initiated BER was increased under hypoxia, as a result of elevated expression of DNA polymerase λ, which was caused by the mTOR kinase. Although, increased BER capacity of hypoxic cells was not promoting their survival or tolerance to DNA damage, re-oxygenated cells were extremely resistant to various types of DNA damage. This suggested that hypoxic preconditioning is a main way to alter BER capacity under changing oxygen availability and consumption. Furthermore, the expression of the four core BER genes, and BER function was uncoupled from the amount of ROS generated or from the content of oxidative lesions in DNA, either during hypoxia, re-oxygenation, or their transitions. Finally, the expression of BER genes and the capacity of BER were elevated in solid tumors of human and animals. Like observed in case of non-transformed cells, upon re-oxygenation, it allowed revived tumor cells to tolerate excessive DNA damage caused by multiple, also therapeutic, agents, and thus determined their *ex vivo* resistance to anti-cancer treatment.

GERMAN

Die Anpassung an eine Welt mit ihren 20% atmosphärischen Sauerstoff ist eng mit der Fähigkeit eines Organismus verknüpft, auch mit seinem schädlichen Potential umzugehen. Die Reparatur der DNA Schäden, die durch sogenannte "Reactive Oxygen Species" (ROS) entstehen, ist sehr wichtig, da eine ungenügende DNA Reparatur zu Krebs führen kann. Die DNA Reparatur, welche die sogenannten 8-oxo-guanine (8-oxo-G) entfernen kann, bezeichnet man als Basenexzisionsreparatur (BER). Diese umfasst 30 verschiedene Proteine, welche zu korrekten Reparatur, sowie zur Verhinderung der genetischen Instabilität beitragen. In den letzten Jahren wurden regulatorische Mechanismen entdeckt, die auf eine Abhängigkeit der BER vom Zellzyklus und der Checkpointkontrolle hindeuten. Hingegen ist nicht bekannt, wie die BER auf hohe Mengen ROS reagiert, wie sie bei erhöhtem Sauerstoffverbrauch der Zelle entstehen (z. Bsp. beim Gewebewachstum und bei der Geweberegeneration). In Gegensatz dazu ist ebenfalls bei der sogenannten Sauerstoffunterversorgung wenig bekannt (z. Bsp. Hypoxie bei Tumoren). Dabei weiß man vor allem noch sehr wenig über deren Regulationsmechanismen.

In dieser Dissertationsarbeit wurden vier wichtige BER Proteine untersucht. Es handelt sich um die beiden DNA Glykosylasen hOGG1 und MUTYH und die beiden DNA Polymerasen λ und β. Sie wurden in Bezug auf Hypoxie mit anschließender Reoxygenierung getestet. Die hOGG1 initiierte BER wurde sowohl unter Hypoxie als auch während der Reoxygenierung massiv stimuliert. Die Kontrolle erfolgte durch den HIF1a Regulationsweg, indem sowohl die mRNA als auch die Proteine Mengen von hOGG1 als auch von DNA Polymerase β induziert wurden. Im Weiteren wurde während der Reoxygenierung die Aktivität von hOGG1 durch Phosphorylierung durch die Proteinkinase B (PKB, Akt) beeinflusst. Phosphorylierung von hOOG1 am Ser51 by Akt2 erhöhte die Aktivität von hOGG1 vermittelter BER während der Reoxygenierung, indem hOGG1 rascher in den Zellkern transportiert wurde. Auf der anderen Site wurde die Aktivität von MUTYH iniziierter BER unter Hypoxie stimuliert. Dies weil die Expression von DNA Polymerase λ als Folge der mTOR Kinase erhöht war. Überraschenderweise waren reoxygenierte Zellen sehr resistent gegen DNA Schäden. Dies deutete darauf hin, dass die Zellen konditioniert sind auf häufigen Wechsel in ihrer Sauerstoffumgebung. Im Weiteren waren die vier erwähnten BER Proteine unabhängig vom der Menge produzierten ROS oder von der Menge der effektiven Sauerstoffschäden, die während der Hypoxie und der Reoxygenierung entstanden. Schließlich wurden in Tumoren von Mensch und Hund erhöhte BER Werte gefunden. Dies deutet darauf hin, dass Tumorzellen nach Reoxygenierung höhere DNA Schäden überleben können und somit eine Resistenz gegen Chemotherapeutika entwickeln können.

I. INTRODUCTION

I.1. OXYGEN HOMEOSTASIS

I.1.1. OXYGEN, HYPEROXIA AND HYPOXIA

Oxygen (atomic number 8, chemical symbol O) is the most abundant chemical element in the biosphere, and is the third element in the universe, after hydrogen and helium. It is the most common element in the Earth's crust (42.9%, by mass) and the second most common component of the Earth's atmosphere, constituting up 20.8% of its volume and 23.1% of its mass (some 1015 million) (Hampel, 1968).

Historically, aerial substance responsible for combustion was first discovered in 1772 by Swedish pharmacist Carl Wilhelm Scheele, and few years later it was isolated and named "oxygen", by the French chemist Antoine Lavoisier (Ephraim et al., 1939). He derived this name from the Greek roots οξύς (oxys) ("acid", literally "sharp", referring to the sour taste of acids) and -γενής (-genēs) ("producer", literally "begetter"), because it was mistakenly thought, at that time, that all acids require oxygen in their composition.

In atmosphere over 99% of the oxygen is the isotope O^{16} that forms the diatomic molecule O_2, a very pale blue odorless and tasteless gas. O_2 molecule exists in two main states. At its ground and inactive state, it is a triplet, having two unpaired electrons occupying two degenerate molecular orbitals that make the double bond stable, and thus the entire molecule low reactive and paramagnetic. In an active high-energy state, O_2 is singlet and has all the electron spins paired which makes singlet O_2 to be highly reactive towards common organic molecules than is molecular oxygen *per se*. In nature, free oxygen, mainly in its singlet state, is commonly formed from water during photosynthesis, using the energy of sunlight (Krieger-Liszkay, 2005), or produced in the troposphere by the photolysis of ozone by light of short wavelength. In the human body free oxygen is also generated by the immune system as a source of active oxygen needed to eliminate invading microorganisms (Wentworth et al., 2002).

Originally discovered as the "life" gas, it was only two centuries later explained how oxygen is involved in life supporting processes, like energy generation via oxidative phosphorylation in mitochondria (Mitchell and Moyle, 1967) and photosynthesis in chloroplasts (Bryant and Frigaard, 2006), further emphasizing its importance for cell metabolism and physiology. Therefore, organs and tissues in the human body also require constant oxygen supply to maintain their function and to balance energy homeostasis.

The oxygen content in the body of a living organism is usually highest in the respiratory system, and decreases along the arterial system, the peripheral tissues and the venous system, respectively. Therefore, the oxygen content is often given as the partial pressure, which is the pressure, which oxygen would have if it would alone occupy the entire volume (Henrickson, 2005). The normal O_2 concentration to which cells in the human body are exposed varies from 21% in the upper airway to 1% at the corticomedullary junction of the kidney (**Table 1**) (Semenza, 2010). The blood is oxygenated in the lung alveoli, where the level of oxygen reaches 13–16%. As the blood is transported through the body, the oxygen levels decrease. Venous blood contains around 5% oxygen and in certain peripheral tissues, oxygen levels are probably substantially lower than that (Webb et al., 2009). Apart of such a broad range of oxygen exposure, the majority of human tissues optimally functions in O_2 concentrations that do not exceed 9%, but also do not go below 2%. Conditions of the high ventilation (hyperoxia) or shortage of O_2 supply (hypoxia) are unfavorable, toxic and stressful.

Table 1: Partial pressures of oxygen in the human body (P_{O_2})

Unit	Alveolar pulmonary oxygen	Arterial blood oxygen	Venous blood oxygen
kPa	14.2*	11-13	4.0-5.3
mmHg	107	75-100	30-40
	* - data from (Henrickson, 2005)		

Hyperoxia is defined as an excess of oxygen in the lungs or other body tissues, which can be caused by breathing air or oxygen at pressures greater than normal atmospheric. The latter is rare physiological condition and is mainly related to medical practice and toxicity in association with oxygen therapy or other rapid exposures to hyperbaric oxygen.

Hyperbaric (high-pressure) therapy is used to treat diseases that impair the body's ability to take up and use gaseous oxygen, e.g. emphysema, pneumonia and certain heart disorders (Jorgensen et al., 2008). In such cases, oxygen can lead to toxicity, which usually begins to occur at partial pressures more than 50 kilopascals (kPa), or 2.5 times the normal sea-level O_2 partial pressure of about 21 kPa. Convulsions and seizures are visible as toxic effects, (**Fig. 1**). In clinical settings, however, this is not a major problem except for patients that are on mechanical ventilators, since the gas supplied through oxygen masks in medical applications is typically composed of 30%–50% O_2 only by volume (about 30 kPa at standard pressure). Finally, stationary of premature babies in incubators containing O_2-rich air was discontinued due to high rate of associated blindness (Jorgensen et al., 2008).

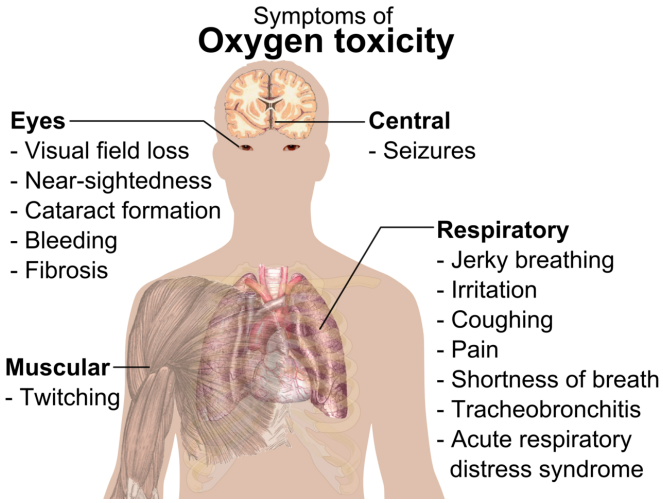

Fig. 1: Main symptoms of oxygen toxicity (Dharmeshkumar N Patel, 2003)

Hypoxia, known as oxygen starvation, in contrast to hyperoxia, is a common physiological and pathophysiological condition. Since it is difficult to define hypoxia with a specific oxygen concentration threshold value so most of current practical definitions consider, hypoxia occurs when oxygen delivery does not meet the demand of a certain organ, tissue or cell (Papandreou et al., 2005). Hypoxia can appear under the following circumstances (Vaupel and Harrison, 2004): 1) the partial O_2 pressure is low in arterial blood due to pulmonary diseases or high altitude (hypoxemic hypoxia); 2) the capacity of O_2 in blood is reduced as a result of anemia, methemoglobin formation, or carbon monoxide poisoning (anemic hypoxia); 3) insufficient blood perfuse to tissues (circulatory or ischemic hypoxia); 4) the diffusion geometry is altered such as increased diffusion distances, concurrent versus countercurrent blood flow within micro vessels (diffusional hypoxia) and 5) intoxication causes cells to be unable to use O_2 as in cyanide poisoning (histotoxic or cytotoxic hypoxia).

For experimental cell cultures, hypoxia is defined as 0.2-2.0% O_2 (usually complemented with 5% CO_2, the rest is nitrogen). At this level the toxicity and growth inhibition to cells are not present, and cellular responses to hypoxia can be measured (Papandreou et al., 2005).

I.1.2. ADAPTION TO HYPOXIA, HIF and mTOR

Once an organism or a single cell is exposed to hypoxia, it may react with switching on mechanisms that regulate responses to changes in oxygen tension and allow adaption to that. These mechanisms are multiple and act dependently on the level of exposure. In response to global hypoxia, such as what occurs at high altitude, the physiology of the entire organism is modified by e.g. increasing the respiration rate, whereas, localized hypoxia, such as at the site of an injury, modifies local cell signaling with confined effects on blood vessel recruitment.

In the mammalian organism specialized chemoreceptor cells, in the carotid and neuroepithelial bodies, sense oxygen. Contributions from mitochondrial production of H^+ and direct membrane effects lead to a reduction in outward potassium current in these cells during hypoxia. The resulting membrane depolarization, the calcium entry and the neurotransmitter release, immediately alter the physiology of the organism by stimulating an increase in respiratory and heart rates (Lahiri et al., 2006).

On the other hand, responses to chronic hypoxia, may take several hours to develop and generally result in widespread changes in gene expression. In general, there are two adaptation choices in chronic hypoxia: reduction of oxygen consumption by switching to an anaerobic metabolism and by decreasing energy usage (i.e., reduced proliferation) or, alternatively, increasing oxygen delivery to cells via increased erythropoiesis and blood vessel formation (Mole and Ratcliffe, 2008).

No matter which mechanisms are involved and how their regulation differs among various organisms or cells, they all work through hypoxia-induced molecules. The hypoxia-dependent generation and regulation of these molecules, to a large extent, are managed at the transcriptional level by hypoxia-inducible factors.

Hypoxia inducible factor (HIF) HIF-1 was originally discovered as a transcription factor inducing EPO transcription in response to hypoxia (Semenza and Wang, 1992). Five years later HIF-2 (also named EPAS-1 or HLF-1) was discovered and showed a close sequence similarity to HIF-1 (Ema et al., 1997) and eventually HIF-3 was reported as a negative regulator of HIF induced transcription (Makino et al., 2001). HIF-1 is still the most studied HIF and increasing research is focusing on HIF-2. Little, however, is still known about HIF-3 (**Fig. 2**).

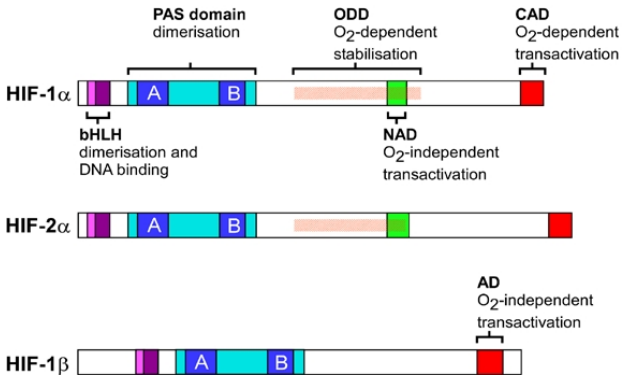

Fig. 2: Domain structure of HIF subunits. Basic structure of the mammalian HIF-1α, HIF-2α, and HIF-1β proteins, showing conserved functional domains including basic helix-loop-helix (bHLH), Per-Arnt-Sim homology (PAS), oxygen-dependent degradation (ODD), and N-terminal transactivation (NAD) and C-terminal transactivation (CAD) domains (Bracken et al., 2003).

The HIFs belong to the basic helix-loop-helix PAS (bHLH-PAS) domain family of transcription factors and are active as heterodimers consisting of one HIF-α subunit and one HIF-β subunit. Under hypoxic conditions HIF-lα and HIF-2α rapidly accumulate and bind HIF-1β (also called ARNT), recruit the transcriptional co-activators p300 and CREB-binding protein (CBP) and binds to hypoxia-response elements (HREs), thus activating the transcription of several hundred of different hypoxia-responsive genes (**Fig. 3**). HIF-1 binding is only detected at genes with increased expression but it can also regulate transcription of many genes in a negative way indirectly by regulating transcriptional repressors and microRNAs (Mole et al., 2009).

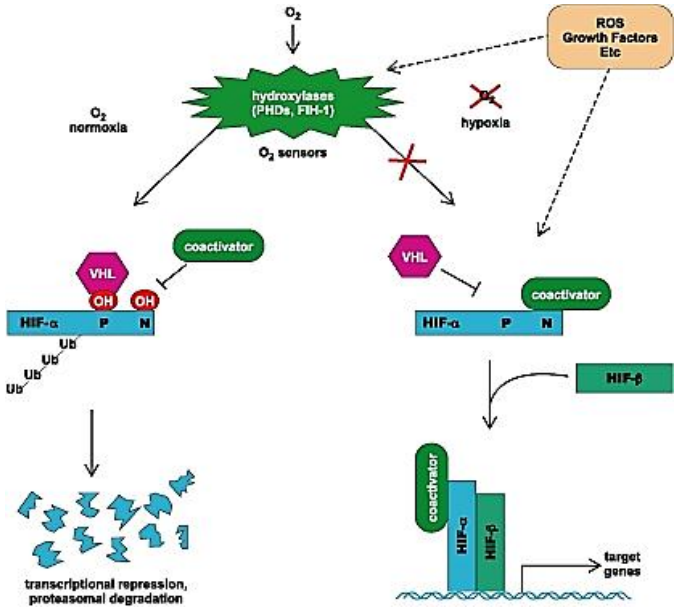

Fig. 3: Oxygen-dependent regulation of HIF-α. Prolyl and asparaginyl hydroxylation of HIF-α modulate the stability and transcriptional activity of HIF-α in an oxygen-dependent manner, and therefore the overall activity of HIF target genes. For details see text and (Bardos and Ashcroft, 2005).

HIF-α subunits are continuously transcribed and translated and its regulation is primarily post-translational (Gordan and Simon, 2007). The expression of HIF-α protein is tightly regulated in an oxygen dependent way (**Fig. 3**). In the presence of oxygen, prolyl hydroxylase domain proteins (PHDs) hydroxylate HIF-α, generating a binding site for the von Hippel-Lindau (VHL) protein, which is a component of an ubiquitin ligase complex. HIF-α is then polyubiquitinylated and thus a target to proteasomal degradation. Another way in which oxygen regulates HIF-α is via the factor inhibiting HIF-1 (FIH-1) protein that can hydroxylate HIF-α, thereby preventing binding of the co-activators p300 and CBP. This results in an inhibition of transcription activation (Kaelin and Ratcliffe, 2008). HIF-α can also be regulated in oxygen-independent ways, e.g. by mutations in the regulating proteins. The most obvious example is mutations in VHL that impair the ability of pVHL to ubiquitinate HIF-α leading to HIF-α accumulation in non-hypoxic cells. These mutations are most common in renal cell cancer and hemangioblastoma (Kaelin and Ratcliffe, 2008).

HIF-α can also be accumulated due to increased transcription and translation. Several different important signaling pathways in tumor cells have been associated with increased HIF-α expression. Different growth factor signaling systems that have been reported to increase HIF-α protein levels include EGFR in prostate cancer (Zhong et al., 2001), HER2 (Laughner et al., 2001) and RAS in breast cancer (Blancher et al., 2001), and VEGF in colon cancer (Calvani et al., 2008). There were several different growth factors reported to up-regulate or stabilize HIF-lα expression (Bardos and Ashcroft, 2004). The effects on HIF-α expression modulated by these different growth factors seem to be dependent on signaling pathways central to the tumor cells, i.e. the PI3K/AKT, the mTOR and the RAS/MAPK pathways (Bardos and Ashcroft, 2004). Differences in HIF-1 and

HIF-2 function are well studied in the context of embryonic development. H1F-1α$^{-/-}$ mouse embryos die in mid gestation due to decreased erythropoiesis and cardiovascular malformations and neural tube defects, indicating that HIF-1 is needed for normal development (Yoon et al., 2006). At the same time, H1F-2α knockout mice models have generated differing phenotypes. Most H1F-2α$^{-/-}$ mice die *in utero* (Compernolle et al., 2002) and the few mouse strains that survived showed multiple organs pathology (Scortegagna et al., 2003) or died during neonatal period due to a severe respiratory distress syndrome (Compernolle et al., 2002). Generalizing these results it seems that most of the H1F-2α knockout mice experienced defects in vascular development of different organs and the different phenotypes may be due to different genetic backgrounds. The discrepancies of HIF-1 and HIF-2 involvement in mouse embryo development can also be explained by the difference in their expression. HIF-lα mRNA was more abundantly expressed and especially high in the myocardium, primitive gut and neuroepithelium in mouse embryonic development whereas HIF-2α mRNA was mostly expressed in the developing vasculature, but also found in lung, kidney, olfactory epithelium and adrenal gland (Carmeliet et al., 1998). In human tissues HIF-lα is expressed ubiquitously, whereas HIF-2α expression has been found to be restricted to endothelium, kidney, heart, lung, liver, pancreas, intestine and brain (Wiesener et al., 2003).

The differences in gene regulation by HIF-1 and HIF-2, respectively, have been investigated in different studies using gene expression microarrays and chromatin immunoprecipitation (ChIP). HIF-1 seems to be the most dominant of the two in most tissues but the regulation is tissue and cell type specific (Lofstedt et al., 2007). Erythropoiesis, angiogenesis and glycolytic metabolism is regulated by several genes and is differently regulated by HIF-α subtype in different cell types (Gordan and Simon, 2007). It seems that HIF-lα is needed for inducing glycolytic enzymes in response to hypoxia whereas HIF-2α can mediate many other hypoxia-responses in angiogenesis, growth and metastasis. Previous studies have not found selective binding of HIF-lα or HIF-2α to specific loci but rather the binding to different loci seemed to be cell type specific. Recently, differences in HIF-lα and HIF-2α regulated gene transcription in the breast cancer cell line MCF-7 was reported, stating that despite a large overlap in HIF-α isoform binding there were substantial differences in gene regulation with HIF-2α contributing very little to the overall HIF response (Mole et al., 2009). It seemed that HIF-lα was the most important regulator for adaptation to acute hypoxia. Differences in HIF-α subunit regulation by time and oxygen levels in neuroblastoma has been reported (Holmquist-Mengelbier et al., 2006), suggesting that HIF-lα is more important in acute hypoxia whereas HIF-2α is more important in response to chronic hypoxia and that HIF-2α is also stabilized at "normal" end-capillary O_2 levels of 5%. On the other hand, HIF-lα and HIF-2α expression patterns differ in tumors. HIF-lα is most often found in perinecrotic zones, representing areas of hypoxia (Helczynska et al., 2003) whereas HIF-2α is also found in perivascular areas that are non-hypoxic (Holmquist-Mengelbier et al., 2006). These findings also imply different roles of HIF-lα and HIF-2α in tumor progression.

At the translational level cells respond to hypoxia, involving mainly, another major regulator – the **mammalian target of rapamycin (mTOR)**. It was discovered as the target for its specific inhibitor, rapamycin, an immunosuppressant and antibiotic, which was isolated from the bacteria *Streptomyces hygroscopicus* (Sehgal, 2003). Using RNA silencing technique it was found that rapamycin does not affect all mTOR functions; suggesting mTOR may be present in different isoforms or complexes. Indeed, two TOR complexes, mTORC1 and mTORC2 have been identified in yeast and later in a variety of eukaryotes (Loewith et al., 2002). mTORC1 is rapidly and specifically inhibited by FKBP12-bound rapamycin but mTORC2 is not acutely rapamycin-sensitive. However, long-term treatment with rapamycin can avoid the formation of complex 2 in some cell types, e.g., in endothelial cells (Sarbassov et al., 2006). The newly synthesized mTOR-protein immediately forms a complex with FKBP12-rapamycin, which prevents association with rictor (Sarbassov et al., 2006). Composition of mTOR complexes defines their function and identity.

Regulatory associated protein of mTOR (Raptor, 150 kDa) and PRAS40 are functional parts of TORC1. MLST8 is also present in mTORC1, but is probably not required for all of mTORC1's functions (Loewith et al., 2002). Formation of mTORC2 requires mTOR to assemble the rapamycin-insensitive companion of mTOR (Rictor) and mSIN1 (mammalian stress activated protein kinase [SAPK]-interacting protein). In mTORC2, mLST8 is a functionally and structurally required component. The mTOR regulatory protein rictor (also known as mAVO3) is a large protein (200 kDa) and contains no obvious catalytic motifs, but repetitive domains, similar to mSIN1 (Loewith et al., 2002) (**Fig. 4**).

mTOR integrates various signals to regulate cell growth. Four major inputs have been implicated in TOR signaling: growth factors, nutrients, energy, and stress (Wullschleger et al., 2006) (**Fig. 5**).

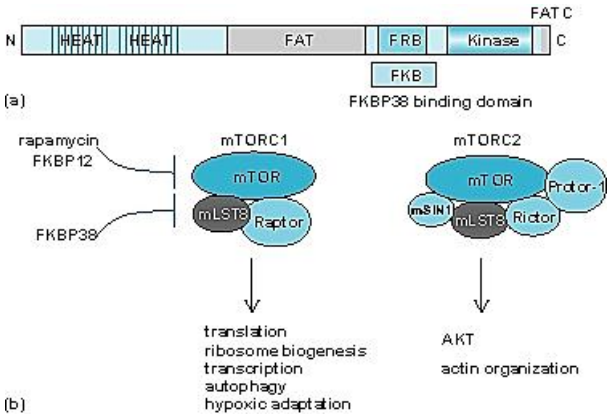

Fig. 4: Schematic presentation of (a) mTOR structure and (b) the mTOR complexes. For details see text (Wullschleger et al., 2006).

In response to environmental stress, such as hypoxia or low energy, cells down regulate energy-demanding processes and arrest growth.

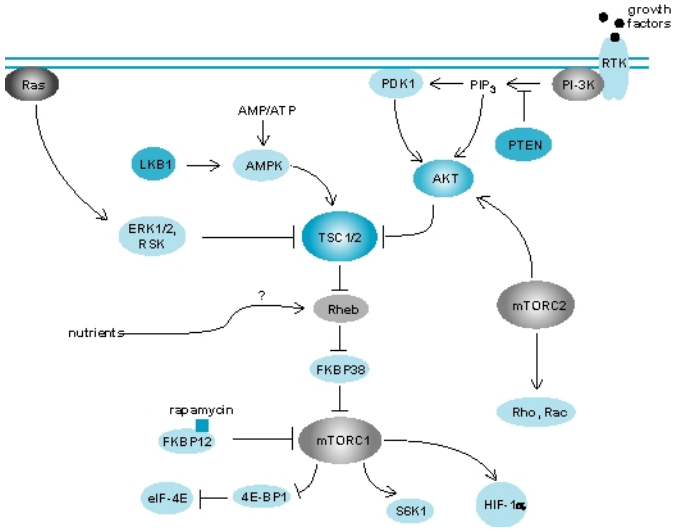

Fig. 5: The mTOR-signaling network. Activation processes are marked by arrows and inhibition by T-bars. For details see text and (Wullschleger et al., 2006).

Upon hypoxia, TOR signaling is inhibited and protein synthesis is thereby down regulated. Hypoxia is transduced to mTORC1 via the two homologous proteins REDD1 and REDD2. The expression of REDD is up-regulated upon hypoxia by HIF-1 (Brugarolas et al., 2004). However, stabilization of HIF-1α under hypoxia has been shown to depend on active mTOR-signaling

suggesting that hypoxia activates mTOR signaling (Hudson et al., 2002). Furthermore, hypoxia has been shown to increase proliferation of lung adventitial fibroblasts, endothelial cells and of angiogenesis *in vitro* in mTOR dependent way (Humar et al., 2002). Therefore hypoxia can inhibit mTOR signaling in some processes and activate it in others.

I.1.3. TUMOR HYPOXIA AND THERAPY RESISTANCE

Balanced oxygen homeostasis is the basis for normal function of a human body as well as their individual cells. Subtle regulation of hypoxic responses helps to adapt to the changes in oxygen availability. Incorrect hypoxic adaptation has been associated with several human diseases, such as congenital polycythemia, vascular ischemic disease and pulmonary hypertension (Semenza, 2011). Among multiple clinical manifestations of disordered oxygen homeostasis, the most notable is coordinated adaptation to hypoxia in cancer, known as **tumor hypoxia**.

Early reports demonstrated that tumors grow in close proximity to blood vessels, and that tumor cells located more than 180 μm away from blood vessels exhibit considerable degree of necrosis (Thomlinson and Gray, 1955). This distance correlates with the diffusion limit of oxygen (100-150 μm), when it passes from the capillary network and is metabolized within tissues. Similar to normal cells and tissues, cancer cells require supply of oxygen and nutrients to maintain membrane transport, chemical synthesis, growth and overall homeostasis. In addition elimination of waste products by the blood circulation is very important. When solid tumors expand in size, their needs for oxygen are also increased. However, the delivery of O_2 to malignant and stromal cells is frequently reduced or even abolished due to insufficient and abnormal angiogenesis (Carmeliet and Jain, 2000), which in addition to anemia that can reduce the capacity of oxygen transport, thus ultimately leading to tumor hypoxia. These processes generate micro regions with very low or even zero (anoxic) oxygen partial pressures, distributed heterogeneously within most solid tumors.

Tumor hypoxia is present when oxygen levels fall below a critical value, leading to a progressive decrease in ATP production. On the other hand, critical oxygen level leading to hypoxia can vary widely among malignant tumors. Assessments of the tumor oxygenation status by both invasive and non-invasive methods indicate that oxygen P_{O_2} of 10 mmHg (corresponding to 1.3% O_2) can be considered hypoxic (Hockel et al., 1996). This cut-off level was found to distinguish hypoxic cervical cancers with poor prognosis, from less-hypoxic corresponding tumors with significantly better survival. Tumors with a $P_{O_2} < 10$ mmHg also exhibit a substantially increased probability of invasion and metastasis (Brizel et al., 1996). Clinical studies reveal that tumor hypoxia is a predictive factor for poor outcome, independent of tumor characteristics such as patient age, tumor size and grade, and the extent of necrosis (Hockel and Vaupel, 2001).

Furthermore, tumor hypoxia is highly associated with increased resistance to anti-cancer therapy. Due to the decreased levels of available oxygen, which is the substrate for the formation of reactive oxygen species (ROS), causing DNA damage by photon therapy, hypoxia impedes radiation treatment (Gray et al., 1953). In addition, hypoxia can confer radiation resistance by increasing the expression of heat-shock proteins, inducing cell growth, or by inhibiting the apoptotic pathway (Graeber et al., 1996). Furthermore, treatment of cancer patients with chemotherapeutic drugs is also hampered by tumor hypoxia, due to several different mechanisms (Brown and Wilson, 2004). The distance of hypoxic cancer cells from blood vessels causes physical inaccessibility of therapeutic agents. Abnormal architecture and insufficient circulation in tumor blood vessels cause higher interstitial pressure, which can be associated with low penetration of drugs. Furthermore, the therapeutic effect of several substances can be dependent on presence of oxygen. Many drugs are also dependent on a high range of proliferating tumor cells for proper function, and since hypoxia is known to be involved in decreasing proliferation, a diminished effect of the drugs might be at hand. In addition, hypoxia mediates an in tumor selection for cells resistant to p53-mediated apoptosis,

thereby desensitizing cancer cells for apoptosis-inducing agents (Graeber et al., 1996). Finally, hypoxia can induce expression of genes providing drug resistance, as exemplified by the *mdr1* gene (Comerford et al., 2002).

I.2. REACTIVE OXYGEN SPECIES AND DNA DAMAGE

I.2.1. ROS AND OXIDATIVE STRESS

Reactive oxygen species (ROS) defines a collective term that can refer to free radical oxygen species that have an unpaired electron in its valence shell. It also refers to certain non-radicals that are either strong oxidizing reagents or easily converted to free radicals (Wiseman and Halliwell, 1996) (**Fig. 6**). The major ROS generated in biological systems include superoxide radical anions ($O_2\bullet^-$), hydroxyl radicals (HO●), peroxyl radicals ($RO_2\bullet$), alkoxyl radicals (RO●), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Wiseman and Halliwell, 1996). Production of ROS can arise from both endogenous and exogenous sources. Different environmental agents including tobacco smoke and air pollutants contain ROS or can generate ROS. Chemical carcinogens (such as benzopyrene, aflatoxin and benzene), ionizing radiation and UV light (UVR-A and UVR-B) can also induce ROS (Loft and Poulsen, 1996).

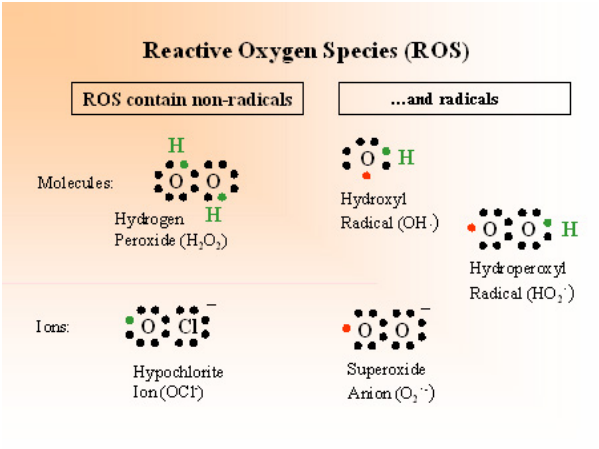

Fig. 6: Different types of ROS (Wiseman and Halliwell, 1996). Unpaired electrons marked as red dots.

In biological systems, a major source of endogenous ROS is the complete reduction of oxygen, via the electron transport chain in mitochondria during cellular respiration and ATP production. A mammalian body produces approximately half of its body weight ATP, mostly through oxidative phosphorylation. Up to 5% of oxygen molecules undergoes one-electron reduction and are converted to superoxide radical anions (Klaunig and Kamendulis, 2004). Mitochondrial superoxide dismutase, an antioxidant enzyme, can catalyze the conversion of excess superoxide radical anions to hydrogen peroxide while catalase and glutathione peroxidase convert hydrogen peroxide to water (Klaunig and Kamendulis, 2004) (**Fig. 7**). Excess hydrogen peroxide can leak from the mitochondria and into the cytosol (Yu, 1994). In the presence of ferrous iron, hydrogen peroxide can decompose to hydroxyl radicals via the Fenton reaction:

$$\text{The Fenton reaction: } Fe^{2+} + H_2O_2 = Fe^{3+} + \bullet OH + OH^-$$

Hydroxyl radicals are also formed in a similar manner when reduced forms of other transition metals such as copper come into contact with hydrogen peroxide. Iron and copper are sequestered in proteins including ferritin, transferrin, caeruloplasmin and metallothionein. Oxidative

stress has been shown to release transition metals from proteins and the released metals can then participate in Fenton reactions to produce hydroxyl radicals (Halliwell and Aruoma, 1991).

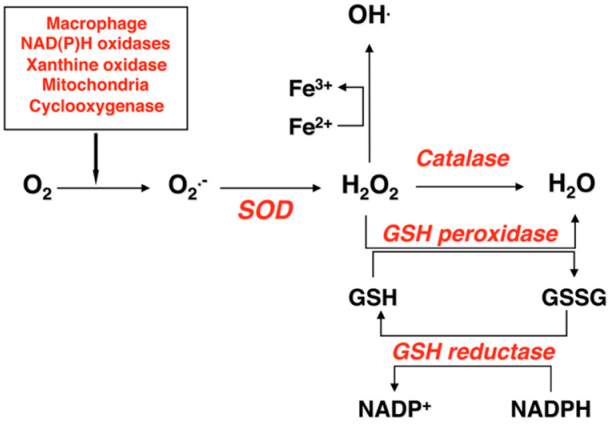

Fig. 7: Generation and detoxification of ROS (Klaunig and Kamendulis, 2004).

Various enzymes that catalyze redox reactions can also contribute to the generation of ROS. During CYP (define abbreviation) catalyzed metabolism, the generation of ROS can arise via different processes. Redox cycling in the presence of molecular oxygen and uncoupling can result in the generation of superoxide anion radical and hydrogen peroxide (Klaunig and Kamendulis, 2004). Several CYPs have the ability to generate ROS during metabolism. CYP2E1 produces a prolonged burst of ROS near the site of substrate oxidation during ethanol metabolism (Ekstrom and Ingelman-Sundberg, 1989). Other enzymes can also produce ROS when catalyzing reactions. Organelles derived from endoplasmic reticulum called peroxisomes are responsible for breaking down fatty acid chains and contain high levels of oxidase enzymes that generate hydrogen peroxide (Yu, 1994). The immune response can generate excess ROS in a process called the respiratory burst. Activated macrophages and hepatic Kupffer cells elicit rapid and transient increase in oxygen uptake to generate superoxide anion radicals and hydrogen peroxide (Klaunig and Kamendulis, 2004). Release of ROS is important for a host's defense and results in localized tissue inflammation and induction of genotoxicity and cytotoxicity in foreign microbes (Yu, 1994).

Oxidative stress is an imbalance between the production of reactive oxygen species and a cell's ability to readily detoxify the reactive intermediates from macromolecules (proteins and fatty acids), or to repair the resulting damage (DNA, RNA). Disturbances in the normal cellular redox state can cause toxic effects through the production of ROS that damage all components of the cell, including proteins, lipids, and, most importantly DNA. This can lead to multiple pathologies, like atherosclerosis, Alzheimer's disease, premature aging and cancer (Valko et al., 2007) (**Fig. 8**).

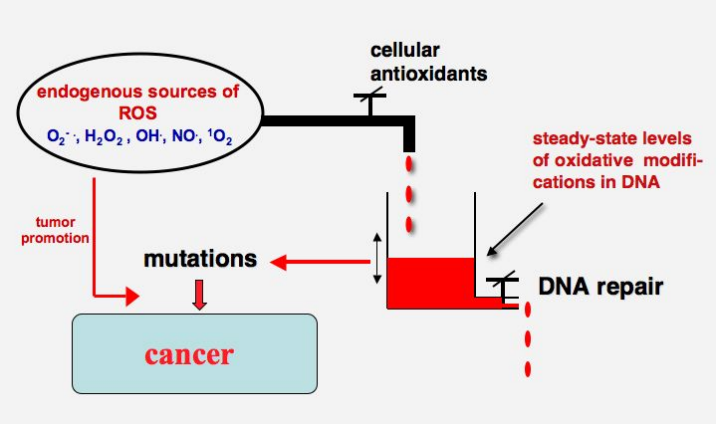

Fig. 8: Schematic representation of oxidative stress and its consequences (Valko et al., 2007).

I.2.2. BASE DAMAGES AND THEIR CONSEQUENCES

Bases in DNA as well as the sugar and the phosphate backbone are constantly damaged. Three of the four bases normally present in DNA (cytosine, adenine, guanine), as well as 5-methylcytosine contain exocyclic amino groups. Spontaneous hydrolytic deamination converts these bases to uracil, hypoxanthine, xanthine, and thymine, respectively (**Fig. 9**), some of which can give rise to mutations during replication and even repair.

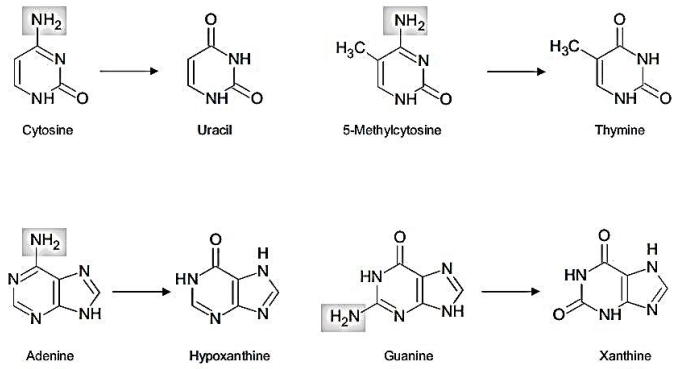

Fig. 9: Products formed from the deamination of bases in DNA (Friedberg and Friedberg, 2006)

Spontaneous loss of bases, mostly purines, occurs at considerable rates that generate the cytotoxic and mutagenic apurinic/apyrimidinic (AP) sites (Nakamura et al., 1998). However, a major source of DNA damage is the attack by reactive oxygen species. ROS can generate mutagenic oxidative base lesions (e.g. 8-oxo-G) and modified bases that can block replication (e.g. thymine glycol) (**Fig. 10**) and strand breaks (Bjelland and Seeberg, 2003).

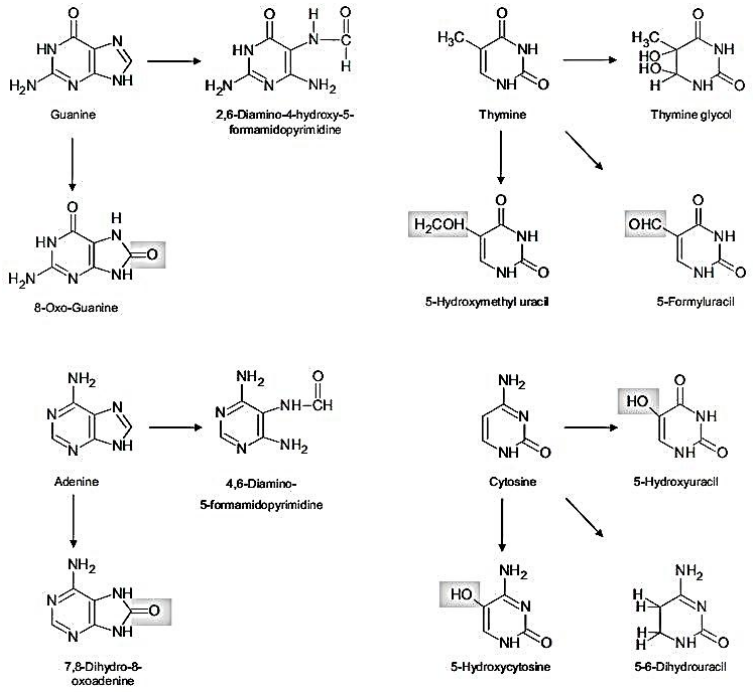

Fig. 10: Common oxidative base damage in DNA (Friedberg and Friedberg, 2006)

Alkylation lesions in DNA result from endogenous compounds, environmental agents and alkylating drugs. Alkylating agents are electrophilic compounds with affinity for nucleophilic

centers in DNA (**Fig. 11**). In general, the ring nitrogen of the bases are nucleophilic, with the N^7 position of guanine and the N^3 position of adenine the most reactive, followed by O^6 in guanine (Friedberg and Friedberg, 2006). Alkylated bases can be mutagenic (O^6-mG) or can block replication (3-meA). Alkylation of oxygen in the phosphodiester linkage results in the formation of phosphotriesters, which apparently are not repaired and are assumed to be relatively harmless (Friedberg and Friedberg, 2006).

The types of DNA damage (base damage or strand break), the location of the lesions in the genome (a promoter region, introns or exons, and actively transcribed regions or transcriptionally silent regions), as well as the cell cycle, and the type of the cells affect the level of mutagenicity or cytotoxicity of DNA lesions.

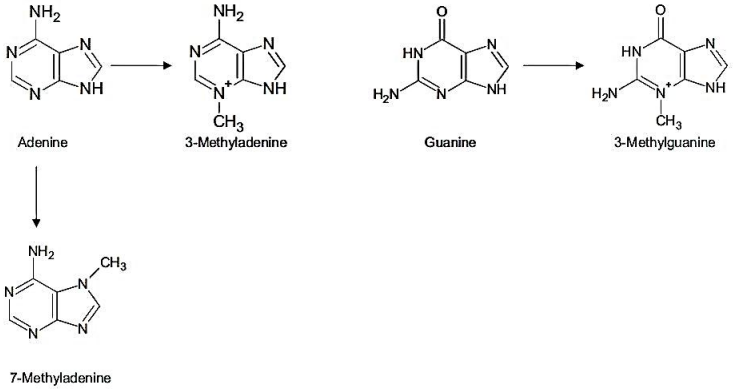

Fig. 11: Products of alkylation damage of bases in DNA (Friedberg and Friedberg, 2006)

Damaged bases are the most frequent form of DNA lesions (Holmquist, 1998), thus, the major consequence of DNA base lesions is the introduction of mutations that could result in the alteration of the genetic information. In this respect, a significant group of DNA base lesions are relatively harmless until they are "fixed" as mutations during replication. Hence, DNA repair is particularly important in actively replicating cells (van Loon and Hubscher, 2009). Accordingly, non-dividing human cells are also known to accumulate base damage in their genome (Nouspikel and Hanawalt, 2002).

The oxidative base damage 8-oxo-guanine (**8-oxo-G**) is one of the most prevalent lesion in the human DNA with an estimated steady state level of 10^3 lesions per cell in normal tissues, and up to 10^5 lesions per cell in cancer tissues (Neeley and Essigmann, 2006). The presence of 8-oxo-G is often used as a biomarker to indicate the extent of oxidative stress (Klaunig and Kamendulis, 2004). The importance of 8-oxo-G results from its high mutagenic potential explained by its strong ability to functionally mimic T, thus, leading to the incorrect bypass of lesion by replicative DNA polymerases (Pols) α, δ, and ε (**Fig. 12**). The incorporation of adenine (A) instead of cytosine (C) opposite the lesion is 10 times more frequently performed by replicative Pols, which consequently might give rise to the CG->AT transversion mutations if A:8-oxo-G mismatches are left unrepaired. A high rate of GC-> TA transversion mutation has been found to be associated and, furthermore, can lead to various cancers (Pleasance et al., 2010).

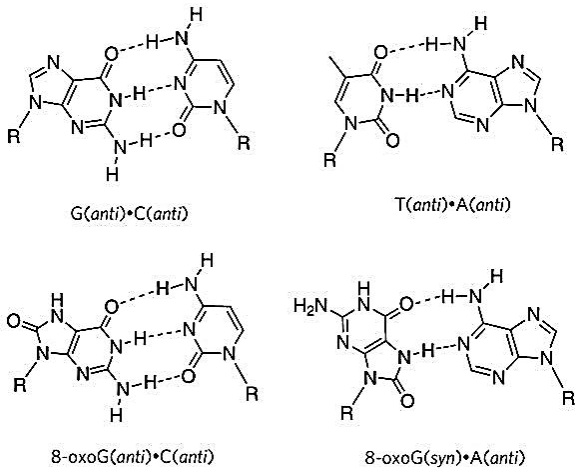

Fig. 12: Structure of 8-oxo-G containing base pairs. The structures of the C:G and A:T base pairs (top) are compared with those of C:8-oxo-G and A:8-oxo-G. 8-oxo-G (bottom) differs from G by an oxo group at C^8 and an NH at N^7. This subtle change allows 8-oxo-G to base pair easily with either A or C (David et al., 2007).

I.3. BASE EXCISION REPAIR

I.3.1. CORE BER PROTEINS AND THEIR FUNCTIONS

Base excision repair (BER) is the predominant DNA damage repair mechanism for the repair of the many base lesions (**Fig. 13**).

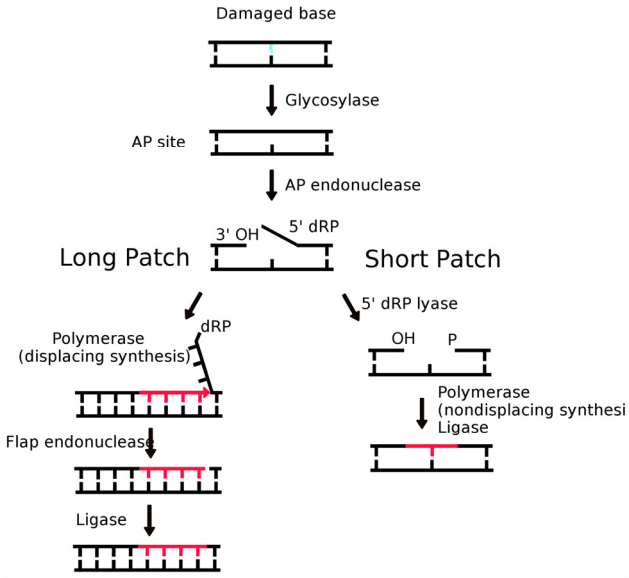

Fig. 13: Simplified schematic representation of the long-patch and short-patch BER pathways (Christmann et al., 2003).

The repair process is initiated with removal of damaged bases by the 11 known lesion-specific DNA glycosylases and completed by either a short-patch or long-patch BER mechanism. Following cleavage of the phosphodiester backbone immediately 5' to the abasic site (AP site) by apurinic/apyrimidinic endonuclease 1 (APE1), via the so-called short-patch BER pathway, Pol β hydrolyzes the resulting 5'deoxyribose phosphate (5'dRP) moiety and inserts a single nucleotide. Alternatively, in the long-patch repair pathway, an overhang of 2 to 12 bases is produced by Pols δ,

ε, or ß as a result of repair synthesis and strand displacement. The DNA overhang is excised by the flap endonuclease 1 (Fen1). Following DNA strand incision, repair synthesis, and end tailoring if necessary, a complex of DNA ligase IIIα (Lig IIIα) and X-ray repair cross-complementing protein 1 (XRCC1) (for short-patch BER) or DNA ligase I (Lig I) (for long-patch BER) complete the repair by sealing the nick in DNA (Almeida and Sobol, 2007). It is currently thought that the majority of BER is carried out via the short-patch pathway (Hou et al., 2007).

DNA glycosylases recognize and remove damaged or incorrect bases by hydrolyzing the N-glycosidic bond between base and deoxyribose sugar. In human cells, 11 different glycosylases have been identified and characterized based on the types of modified bases removed (Jacobs and Schar, 2012). 8-oxo-guanine glycosylase 1 (**OGG1**) recognizes and releases 8-oxo-G paired with cytosine. Adenine-DNA glycosylase (**MUTYH**) identifies and removes 8-oxo-G lesions that have incorrectly paired with adenine during DNA replication (Cooke et al., 2003). Glycosylases have been further divided into 2 major subtypes, type I and type II. Type I glycosylases identify and remove modified bases leaving an apurinic or apyrimidinic site (AP) in DNA (Jacobs and Schar, 2012). Type II glycosylases recognize and remove damaged bases, but can also cleave the DNA phosphodiester backbone by endogenous 3'-endonuclease activity, giving rise to a single strand break (Jacobs and Schar, 2012). In case of type I glycosylases, the phosphodiester backbone of DNA is cleaved by a separate AP endonuclease that can result in 5'-deoxyribose-5-phosphate (5'dRP) and 3'-hydroxyl DNA termini (Fortini and Dogliotti, 2007). After removal of the damaged base, depending on the type of BER, a single nucleotide or series of nucleotides will be inserted, are inserted by **Pols**.

In short-patch BER, **Pol β** exerts lyase activity and catalyzes the release of the hemiacetal form of 5'dRP formed at the DNA terminus of the AP site (Christmann et al., 2003). The resulting gap is filled by a single nucleotide by **Pol β**. **Lig IIIα** participates in short patch repair and seals the DNA backbone (Christmann et al., 2003). Both Lig IIIα and Pol β have been shown to bind with the scaffolding protein X-ray repair cross-complementing protein 1 (**XRCC1**) for added stability and function (Caldecott et al., 1994) (Robertson et al., 2009). The net result of short patch repair is replacement of the damaged base by a single nucleotide.

In long-patch BER, Pol β first inserts a single nucleotide at the AP site (Fortini and Dogliotti, 2007). After **Pol β** dissociation, further DNA synthesis is done by **Pols δ** and **ε**, resulting in a longer repair patch (Christmann et al., 2003). Proliferating cell nuclear antigen (**PCNA**) and replication factor C (**RF-C**) are recruited to stabilize Pols δ and ε during nucleotide insertion (Stucki et al., 1998). **Fen1** cleaves the original 5'dRP flap structure (Klungland and Lindahl, 1997). PCNA forms a complex with Fen1 to stimulate its endonuclease activity. Finally, **Lig I** interacts with PCNA to seal the DNA backbone (Srivastava et al., 1998). The net result of long-patch BER is replacement of the damaged base by a series of nucleotides, 2 to 12 nucleotides in length.

Other **accessory proteins** may have a role during BER. The addition of purified p53 protein to cell free extracts or reconstituted BER pathway stimulates BER *in vitro*. Conversely, immunodepletion of p53 from cell extracts and deficient p53 cell lines have decreased BER activity (Achanta and Huang, 2004). The p53 protein has been shown to directly bind and stabilize AP endonuclease and OGG1 glycosylase to enhance BER activity (Achanta and Huang, 2004). Furthermore, p53 has been shown to stabilize the interaction between Pol β and DNA termini at AP sites (Zhou et al., 2001).

I.3.2. REGULATION OF BER AND INVOLVMENT OF O_2

The BER pathway is composed of over 30 different proteins, and thus, is required to be subtly regulated in order to maintain its function, since even mere deregulation of some of the

components may lead to defects in DNA repair and contribute to the development of human diseases (Valko et al., 2007).

Posttranslational modifications (PTM) (**Fig. 14**) of BER proteins have been thoroughly investigated during the last few years and multiple published reports suggest that PTMs are the most rapid and reliable way to alter stability and enzymatic activities of core proteins in the pathway (Dianov et al., 2011).

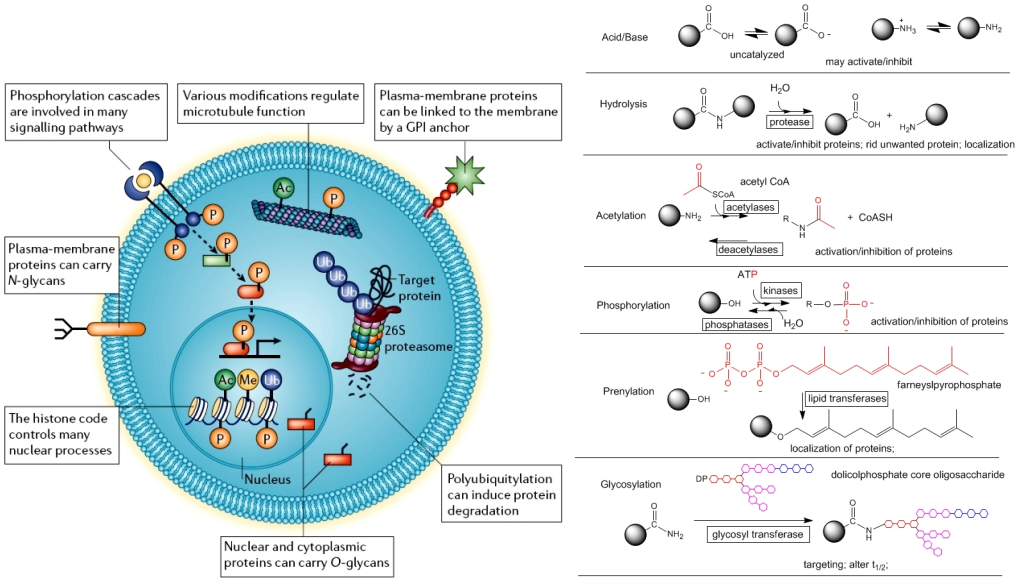

Fig. 14: Posttranslational modifications (PMTs) of proteins (left) and their chemical nature (right). For details see text and (Jensen, 2006).

All core BER proteins studied in this thesis – hOGG1 (Dantzer et al., 2002), MUTYH (Parker et al., 2003), Ape-1 (Yacoub et al., 1997), DNA pol λ (Frouin et al., 2005), Pol β (Luo et al., 2007) -, have been reported to be phosphorylated in response to different stimuli, including oxidizing agents and tobacco smoke (Deslee et al., 2010). Moreover, in case of hOGG1, e.g., phosphorylation affected its cellular localization and enzymatic activity (Dantzer et al., 2002). Alternatively, ubiqutination, has been reported to affect stability of Pol β (Parsons et al., 2008), Pol λ (Wimmer et al., 2008) and MUTYH (Markkanen et al., 2012), thus allowing for rapid accumulation and recruitment of the enzymes to chromatin, in case of DNA damage. In addition, multiple BER genes have been detected to carry in their promoter sequences, recognition sites for different transcription factors (Dhenaut et al., 2000), allowing additional degree of flexible regulation.

Even though many data were generated in the past decade, the detailed mechanisms of BER regulation are still unclear. This mainly concerns the role of O_2 in regulation of BER genes, where the current knowledge is still limited to occasional findings that might suggest a link between BER genes and O_2. The activity of Pol ζ has been reported to be important for cultured mouse fibroblasts at a reduced oxygen concentration (2%), which is believed to partially mimic conditions of embryonic development (Lange et al., 2012). At the same time, exposure of spheroid culture of human skin fibroblasts at different O_2 concentrations, to a low dose of ionizing radiation (IR) identified BER genes among induced targets (von Neubeck et al., 2012). Earlier reports trying to elucidate cellular functions of Pol ι revealed its dependence on HIF-1α activity (Ito et al., 2006). In addition, overexpression of several BER genes has been detected in 30% of 70 cases representing 15 major human cancers (Albertella et al., 2005), including metastatic melanoma cells (Sarasin and Kauffmann, 2008). This tumor has been well known for its hypoxic microenvironment (Pouyssegur et al., 2006), which is also reported to correlate with its treatment resistance (Wirthner et al., 2008).

II. THESIS AIMS

Although, it has been reported that BER enzymes are overexpressed in several tumors, neither their expression nor their role in tumorgenesis has been thoroughly studied. Moreover, how extensive DNA damage through continuous exposure to reactive oxygen species (ROS), generated in excessive oxygen consumption of, or contrary, deprivation of oxygen (like tumor hypoxia), affects the expression of BER proteins and which mechanisms regulate that, has not been clearly defined.

The specific aims of this thesis were:

II.1. To obtain information about the expression of BER genes, in particular the two BER Pols β and λ, the two DNA glycosylases OGG1 and MUTYH and the endonuclease APE-1, in dependence of O_2 availability (normoxia, hypoxia, and hyperoxia);

II.2. To identify pathways, and key factors, regulating expression of BER genes in dependence of O_2 availability;

II.3. To investigate the biological role of O_2 regulated expression of BER proteins;

II.4. To investigate the biological role and impact of O_2 regulated expression of BER proteins on tumor genesis and therapy

II.5. To analyze the expression of BER genes in human and animal tumors according to their grades and oxygenation status.

III. ORIGINAL MANUSCRIPTS

III.1. "Hypoxia Activates the Base Excision Repair Machinery Promoting Cell Survival and Tumor Therapy Resistance"

Mykhailo Razumenko, Daniel P. Stiehl, Simon G. Wrann, Natalie Hofer-Inteeworn, Carla Rohrer Bley, Roland H. Wenger and Ulrich Hübscher

This manuscript describes how differential oxygenation affects expression of BER genes, including the mechanisms required that control it. Furthermore their possible biological implications at the cellular and tissue functions are addressed.

The manuscript is currently *submitted*

Hypoxia Activates the Base Excision Repair Machinery Promoting Cell Survival and Tumor Therapy Resistance

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Running title: O_2 switching activates BER promoting cell survival

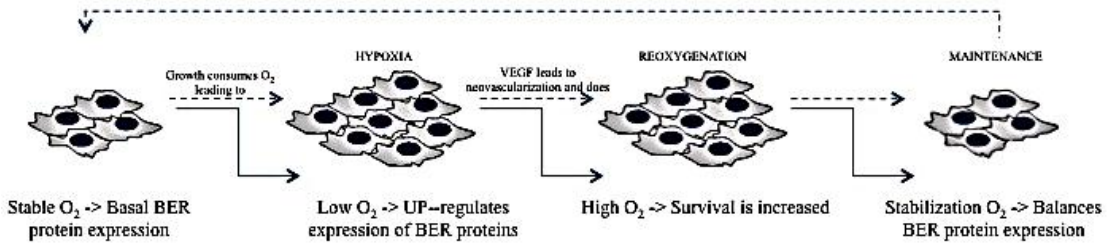
Summary

Base excision repair (BER) is an essential pathway required to correct oxidative DNA damage induced by reactive oxygen species (ROS). Subtle regulation of the pathway is critical to effectively combat mutagenesis and cancer. However, details of this process and its role in tumor genesis remain unclear. Here we demonstrate that hypoxia activates expression of core BER genes leading to an elevated BER functionality, which is dependent on signaling through hypoxia inducible factor (HIF), but not on the content of oxidative DNA lesions and/or production of ROS. Increased BER capacity permits excessive stress tolerance by healthy and neoplastic cells, thus promoting their survival. Finally, activation of BER in solid tumors determines their resistance to anti-cancer therapies.

Significance

Adaptation to a world with 20% O_2 in its atmosphere is closely linked to the ability of cells to couple with oxygen's harmful potential. DNA damage by reactive O_2 derivatives is a major issue, since the insufficient elimination of oxidative lesions from DNA is deleterious and leads to cancer. The mechanism of BER activation described here, explains how repair of oxidized DNA is adjusted to demands and availability of O_2, especially if its concentration and use are rapidly changed, thus resulting in oxidative stress. Increased DNA repair capacity promotes the survival of cells and allows excessive stress tolerance, but, unfortunately, tumors can also exploit this mechanism, thereby developing treatment resistance. Thus, this new aspect of O_2 physiology not only extends our knowledge but can also be the basis for better cancer treatments.

Graphical Abstract



Highlights

- Hypoxia up-regulates BER genes increasing the DNA repair capacity of a cell
- The HIF pathway, but not the amount of ROS or base damages, regulates core BER genes
- Elevated BER functionality increases the cell survival due to genotoxic stress tolerance
- Activation of BER in tumors contributes to their therapeutic resistance

Introduction

Oxygen, which constitutes 20% of the earth's atmosphere, is the most abundant element in the human body (65%) (Maltepe and Saugstad, 2009). Molecular oxygen (O_2) is indispensable for aerobic organism to produce energy by oxidative phosphorylation in the mitochondria. In this process the reduction of O_2 to water by the mitochondrial electron transport chain enables the conversion of adenosine diphosphate (ADP) into adenosine triphosphate (ATP), a universal form of intracellular energy source. The consequence of this reaction, is the formation of reactive oxygen species (ROS) which in spite of their physiological value can also lead to toxic effects, upon accumulation (Bertram and Hass, 2008). Consequences of these accumulations are diseases such as cancer and neurodegeneration as well as premature aging.

ROS are constantly produced in the cellular metabolism and account for up to 95% of all radicals affecting eukaryotic cells through their life cycle. Additional 5% are encountered from exogenous sources, like UV irradiation and environmental pollution (Castro and Freeman, 2001). The majority of ROS molecules are detoxified via cellular antioxidant defense systems generating H_2O as final and secondary oxidants, HO, O_2^-, and H_2O_2 as intermediate substances. The latter three have very short half-lives and are crucial for phagocytic defense of invading microorganisms and intracellular signaling. Concurrently, they are able to damage macromolecules due to their high reactivity, when produced in excess or ineffectively eliminated. This status is known as oxidative stress (Valko et al., 2007).

The most affected targets of ROS are lipids and proteins. Highly abundant and rich in reactive groups they take up most of induced damage thus protecting the least durable cellular components. Besides, the remaining portion of ROS is still able to cause significant harm by oxidizing nucleic acids, in particular DNA, and by impairing the integrity of genetic information, which is a prerequisite for many cancers (Pouyssegur et al., 2006).

Among the various oxidative DNA damages induced by ROS, the oxidation of bases is the most prevalent type. The base guanine (G) is particularly susceptible to oxidative stress. Due to its low redox potential, many oxidized forms of G are present in the cell. 7,8-dihydro-8-oxoguanine (8-oxo-G) is a prevalent lesions in the human DNA with an estimated steady state level of 10^3 lesions per cell in normal tissues, and up to 10^5 lesions per cell in cancer tissues (Neeley and Essigmann, 2006). The importance of 8-oxo-G results from its high mutagenic potential explained by the incorrect bypass of lesion by replicative DNA polymerases (Pols) α, δ, and ε. The incorporation of adenine (A) instead of cytosine (C) opposite the lesion is 10 times more frequently performed by replicative Pols, which consequently might give rise to the CG->AT transversion mutations if A:8-oxo-G mismatches are left unrepaired and possibly leading to various types of cancers (Lange et al., 2011).

In unperturbed cells, base excision repair (BER) is the essential pathway to effectively combat the mutagenic potential of 8-oxo-G (Zharkov, 2008). The main axis, handling over 75% of all 8-oxo-G repairs, is initiated by a damage specific glycosylase, 8-oxo-G DNA glycosylase (OGG1). Human OGG1 recognizes 8-oxo-G lesions flips the bases out and excises them from the DNA, leaving substrates for an endonuclease called apurinic/apyrimidinic endonuclease 1 (APE-1). APE-1 generates free bases filled by DNA pol β. The 5' end is trimmed by the dRPlyase activity of DNA pol β and the product finally

is ligated by DNA ligase III/XRCC1 (Svilar et al., 2010). If an incorrect adenine (A) is incorporated during DNA replication, repair is initiated by the MUTYH glycosylase (MYH) that excises, the A opposite the 8-oxo-G lesion, while APE-1 incises the DNA. Then DNA pol λ incorporates the correct C and performs strand displacement by one base. Finally, the flap endonuclease 1 removes the one nucleotide flap and DNA ligase I seals the DNA thus preventing replication errors introduced by 8-oxo-G in duplicating cells (van Loon and Hubscher, 2009).

Nevertheless, repair of oxidative lesions via BER is not a simple process. Depending on cellular dynamics and extracellular environment, the BER machinery may involve an armamentarium of up to 30 different proteins assisting its function (Mitra et al., 2002). Therefore, a subtle regulation of BER proteins is an issue arising from its complexity. The essential nature of BER in this aspect urges a cell to keep its components active only when needed in order to guarantee the genomic stability required to prevent cancer (Maynard et al., 2009).

The most important cellular condition that requires the adjustment of BER activity is oxidative stress. The inability of cells to couple with the excess of ROS can consequently lead to oxidative damage. Almost 80% of intracellular ROS are generated through oxidative phosphorylation, which is driven by partial O_2 reduction in mitochondria, thus shifts in O_2 availability and consumption are the primary cause of oxidative stress under physiological conditions. This is the reason why all eukaryotic organisms maintain an oxygen homeostasis. Apart from some exceptions, most of human tissues optimally function in O_2 concentrations that do not exceed 9%, but also do not go below 2%. Both of them – the higher tissue oxygenation (hyperoxia) or shortages of O_2 supply (hypoxia) – are conditions of stress (Semenza, 2010). At the cellular level, the family of the hypoxia inducible transcription factors (HIF) is the main mediator of O_2 adaptation. The stabilization of HIFs under hypoxia activates transcription of downstream genes and exhibits cell protective effects, until O_2 supply is restored (Semenza, 2007). On the other hand, mechanisms to protect and repair the DNA of a cell working at higher O_2 concentrations, like BER, are still poorly understood (van Loon et al., 2010).

Unfortunately our current knowledge of the regulation of BER genes, depending on tissue oxygenation, is still limited to occasional findings that might suggest a link between BER genes and O_2 in general. Hence, the activity of DNA pol ζ has been reported to be important for cultured mouse fibroblasts at a reduced oxygen concentration (2%) which is believed to partially mimic conditions of embryonic development (Lange et al., 2012). At the same time, exposure of spheroid culture of human skin fibroblasts at different O_2 concentrations, to a low dose of ionizing radiation (IR) identified BER genes among induced targets (von Neubeck et al., 2012). Earlier reports trying to elucidate cellular functions of DNA pol ι revealed its dependence on HIF-1α activity (Ito et al., 2006). In addition, overexpression of several BER genes has been detected in 30% of 70 cases representing 15 major human cancers (Albertella et al., 2005), including metastatic melanoma cells (Sarasin and Kauffmann, 2008). This tumor has been well known for its hypoxic microenvironment (Pouyssegur et al., 2006), which is also reported to correlate with its treatment resistance (Wirthner et al., 2008). In summary, these findings have urged us to carefully investigate the regulation of BER genes depending on oxygenation, in non-transformed and tumor cells and tissues, focusing not only on its mechanistic insights but also on the biological effects. We demonstrate that the shift in tissue oxygenation (O_2 switch) activates expression of core BER

genes leading to an elevated BER functionality, which is dependent on signaling through HIF, but not on the content of oxidative DNA lesions and/or production of ROS. Increased BER capacity permits excessive stress tolerance by primary and tumor cells promoting their survival. Finally, activation of BER in solid tumors also determines their resistance to anti-cancer therapies.

Results

O_2 switching activates base excision repair proteins via the HIF and the mTOR pathways.

In order to analyze the expression of BER genes in response to different concentrations of O_2, we probed MRC5 human fetal fibroblasts (Jacobs et al., 1970), as a cell line that closely represents physiology of non-transformed cells, for mRNA and protein levels of the 5 core BER genes – DNA pol λ (POLL), DNA pol β (POLB), hOGG1, MUTYH and Ape-1 (Fig. 1 and Fig. S1). mRNA levels of POLB and hOGG1 were increased 6.6-fold and 3.7-fold respectively, remained significantly elevated, and gradually declined for the next 120 minutes, following exposure to 0.2% O_2 for 16 hours. At the same time, mRNA levels of other genes were not affected, except the two hypoxia markers – glucose transporter 1 (GLUT1) and carbonic anhydrase 9 (CAIX) (Hoskin et al., 2003), increasing under hypoxic conditioning raised 8.7 and 13.8-fold, respectively. GLUT1 and CAIX levels returned to basal after 120 min of re-oxygenation (Fig.1, A). Similarly, the levels of hOGG1 and POLB have been found to be significantly (2.5- and 8.1-fold, respectively) elevated during hypoxia, but in contrast to the mRNA pool, the increased protein level of POLB was not maintained during the re-oxygenation phase (Fig. 1, B and C), suggesting its primary transcriptional regulation in addition to proteasome dependent degradation (Wimmer et al., 2008). Remarkably, the expression of MUTYH and APE-1 were not changed as could be expected from its crucial importance for replicative cells, and for the lesion independent removal of glycosylase processed bases. In addition, the protein levels of POLL were also boosted by hypoxia (Fig. 1, B and C), suggesting its regulation to be translational in addition to the post-translational modifications recently described by our laboratory (Markkanen et al., 2012). Since we initially analyzed the expression of core BER genes under stress conditions, we also were curious to see the effects of more physiological oxygen changes, e.g. from 5% to 1%. No differences to the pattern of expression of core BER genes discovered before could be observed. However, the modulation of expression in this case was less pronounced, but still remaining distinctive (Fig. S1, A). In addition, the expression of VEGF, bFGF, TGF-β and GLUT1, that are a part of tissue adaptation to hypoxia, was stimulated by hypoxia under these conditions (Fig. S1, B).

These various responses of BER genes to hypoxia and re-oxygenation prompted us to look closer at possible regulators. HIF-1α has been identified as the primary transcription factor driving hypoxic responses and regulates, besides DNA repair genes, the expression of over 300 other genes. HIF-1 binds to hypoxia response elements in promoters or enhancers of target genes and induces their transcription (Wenger et al., 2005). By using an *in silico* approach (Fig. S1, C) we identified putative HREs in promoters of hOGG1 and POLB genes, but not in the promoters of other BER genes and the control genes (Fig. S1, D). The functional ability of HREs to stimulate gene transcription was demonstrated by reporter gene assay using constructs carrying mutated or wild type HRE sequences (Fig. S1, E). To test

whether the HIF pathway can also regulate BER genes *ex vivo*, we designed short-hairpin RNA (shRNA) constructs and transfected HEK293 cells to produce cell lines with stable HIF-1α or HIF-2α knock-down. Subjected to hypoxia, cells with down-regulated HIF-1α were unable either to induce mRNA (Fig. 1, D) and protein (Fig. 1, E) levels of hOGG1 and POLB or to maintain them during re-oxygenation. This was in contrast to parental HEK293 cells and cells with down-regulated HIF-2α. On the other hand, the expression of other BER genes was affected by either of the two HIFα isoforms under hypoxia, which was also experimentally controlled by evaluating expression of the two hypoxia markers (GLUT1 and CAIX) and the HIF factors (Fig. 1, D and E). Since protein levels of POLL were HIF independently induced under hypoxia (unpublished data), we next probed the mammalian target of rapamycin (mTOR) kinase (Fahling, 2009), a second major modulator of gene expression under hypoxia, for its effect on POLL gene expression. The activated mTOR kinase is known to interfere positively with the translation of downstream target proteins, and can be specifically inhibited by rapamycin and by the cell density (Limoli et al., 2004). To avoid a possible influence of HIF-1α, we treated HEK293 cells stably transfected by shRNA HIF-1α with rapamycin (Fig. S1, G) or plated the cells at defined densities (Fig. S1, H). In both cases elevated protein levels of POLL under hypoxia were observed, while protein levels of MUTYH were not affected and as a positive control the levels of phosphorylated p70S6K kinase, the downstream target of mTOR, was activated by this treatment. At the same time mRNA levels of POLL and MUTYH were not changed due to rapamycin treatment, excluding a possible transcriptional effect (Fig. S1, F).

Taking into account that BER is an orchestra of enzymatic reactions, we next asked whether changes in the expression of BER genes induced by differential exposure to O_2 have consequences on BER functions. To do this we used hairpin oligonucleotides previously designed in our laboratory (van Loon and Hubscher, 2009) (Fig. 1, F) that mimic different situations of 8-oxo-G repair (Fig. 1, G). The MUTYH - POLL axis of BER was active under hypoxia, as detected by the increased [α-P^{32}] dCTP incorporation for substrate 1 (S1), however under re-oxygenation conditions, and at the beginning of incubation, such activity could not be detected (Fig. 1, H) In contrast, the hOGG1 - POLB mediated BER was elevated at the beginning of incubation, and even more under hypoxia and during re-oxygenation, as detected by the increased [α-P^{32}] dGTP incorporation for substrate 2 (S2) (Fig. 1, H). Furthermore, the activity of hOGG1 - POLB mediated BER was dependent on HIF-1α as detected by 10-fold reduced [α-P^{32}] dGTP incorporation for S2 when whole cell lysates of HEK293 cells transfected with HIF-1α shRNA were used (Fig. 1, I). Up-regulated expression of hOGG1 and POLB in this case controls adjustment of the oxidative DNA repair capacity depending on the O_2 availability, while MUTYH – POLL maintains BER under prolonged or chronic hypoxia, known to generate additional amounts of ROS.

The activation of base excision repair genes relies on hypoxia but neither on the extent of ROS production nor on the accumulation of oxidative DNA lesions.

As a next step, we tried to understand how hypoxia and re-oxygenation influences ROS production and accumulation of oxidative damage, and how the latter affects the expression of BER genes. To address this, rates of intracellular ROS formation in cells exposed to different O_2 concentrations were determined through dichlorofluorescein (DCF) oxidation (Fig. 2, A). We observed an over 3-fold reduced rate of ROS formation after 16 hours of hypoxic incubation as compared to the rates measured in cells cultured at 20% O_2.

ROS formation reverted during 120 min of re-oxygenation and increased 6-fold (Fig. 2, B) to levels comparable to those induced by 1 mM H_2O_2 treatment (Fig. S2, A). The accelerated ROS formation was also inhibited under reductive conditions of 1 mM dithiothreitol (DTT) (Fig. S2, A).

The dependence of ROS formation on O_2 concentration almost mirrored the content of 8-oxo-G in the DNA, which was assessed first by immunofluorescence (Fig. 2, C), second confirmed by flow cytometry (Fig. S2, B) and third observed by Southern hybridization combined with immunoblot detection of 8-oxo-G lesions using a specific antibody (Figure S2, C). The accumulation of 8-oxo-G in DNA was found to be 2-fold lower in hypoxia as compared to re-oxygenated or cells cultured in 20% O_2 (Fig. 2, D), suggesting the induction and the accumulation of oxidative lesions to be in linear dependence to O_2 availability. Remarkably, 8-oxo-G mainly accumulated in the cytoplasm during re-oxygenation and before start of hypoxic incubation. This is in contrast to its nuclear accumulation upon the treatment with chemical oxidants like H_2O_2. Furthermore, by using a MitoTracker® probe, 8-oxo-G was also partially localized to mitochondria in the cell (Fig. S2, D), suggesting a possible role of mitochondrial damage in the regulation of BER function.

This finding was in contrast to the previously observed activation of BER genes, since rather reduced than elevated expression levels of BER genes under hypoxia might be expected. We therefore next addressed whether the content of oxidative lesions in DNA, - like 8-oxo-G or signaling via HIF-1α, - is a primary and sufficient event or if their combination is required to modulate expression of BER genes accordingly to O_2 availability. To test this we treated cells with dimethyloxalylglycine (DMOG), a potent inhibitor of prolyl-4-hydroxylation-dependent degradation of HIF-1α, mimicking hypoxia (Asikainen et al., 2005). Applying DMOG we aimed to activate HIF signaling without interfering with accumulation of oxidative DNA damage. Indeed, treatment of cells with 1 mM DMOG for 16 hours induced mRNA (Fig. 2, E) as well as protein levels (Fig. 2, F and G) of HIF-1α target genes under 20% O_2 but with no changes in the 8-oxo-G content between DMOG treated and control cells (Fig. 2, H, left quadrant). Application of 1 mM DMOG also resulted in HIF-1α accumulation (Fig. 2, H) indicating response to hypoxia, as also documented in the activation of the CAIX gene (Fig. S2, E). At the same time, as expected, expression of the HIF independent BER genes, POLL and MUTYH, was not affected by DMOG treatment (Fig. 2, G and Fig. S2, F). In summary, these data suggest that activation of core BER genes relies on HIF signaling rather than on accumulation of oxidative lesions.

Activation of base excision repair genes leads to an increased cell survival in response to the oxidative stress

A crucial part of our study was to relate the hypoxia activated expression of BER genes to its biological role, since the activation of energy demanding machinery under unfavorable conditions is deadly to a cell unless it is absolutely required. It has been reported that some BER genes are important for accelerated tissue growth and proper embryonic development (Ufer et al., 2010). Other DNA repair factors have been assumed to be associated with the ability of hypoxic tumors to resist therapy, that is the cause of over 70% of all anti-cancer treatment failures (Zhou et al., 2006). In this context we checked whether the activation of BER genes under hypoxia might bring a survival advantage to the cells. Using colony formation assays (CFA), we observed that the amount of colonies formed 7 days after treatment of hypoxic cells or cells cultured at 20% O_2 with 1 mM H_2O_2 for the last

2 of 16 hours of incubation were not significantly different (Fig. S3, A), indicating that hypoxic cells are not more tolerant to DNA damaging agents than normoxic cells. In contrast, when H_2O_2 was applied during 120 min of re-oxygenation, only cells previously subjected to hypoxia and activated BER machinery, were able to form a significant number of colonies compared to control cells that were kept constantly at 20% O_2 (Fig. 3, A). The ability of cells to tolerate excessive stress after the re-oxygenation was not limited to H_2O_2 but also to 100 nM methyl methanesulfonate (MMS) (Fig. S3, B) treatment or γ-irradiation with a single dose of 3 Gy (Fig. S3, C). The sequential silencing of core BER genes by specific siRNA identified hOGG1 and POLB genes to be necessary for cells to exhibit protective effects of the activated BER (Fig, 3, C-F). In order to exclude a possible effect of the cellular anti-oxidant defense systems on cell survival during re-oxygenation, we tested the enzymatic activity and protein levels of the two key detoxifying enzymes catalase (Fig. S3, E and F) and GSH peroxidase (Fig. S3, E and G-I). Neither the protein levels nor their catalytic activities were affected by hypoxic preconditioning. In addition, there was only a moderate change in mitochondrial metabolism as measured by the MTT assay (Fig. S3, D). In summary, these data suggest that hypoxic preconditioning activates the expression and function of core BER genes, which in turn provide cells with the ability to tolerate excessive oxidative stress, thus promoting survival.

Activated base excision repair genes can be identified in dog tumors

As mentioned above, certain BER genes were identified to be up-regulated in tumors (Albertella et al., 2005). The main pitfall of these previously published studies was lack of healthy tissue controls while analyzing BER genes in tumors. In order to address this question, we examined matched tumor and healthy tissue biopsies from dogs with spontaneous tumors. This allowed us to precisely estimate mRNA and protein levels for the five core BER genes (hOGG1, MUTYH, APE1, POLB and POLL). We tested eight biopsy pairs of canine non-Hodgkin, non-metastatic stage IIB lymphoma (NHL, stage IIB), for the expression of the five core BER genes in correlation to the average content of $CD20^+$ B-cells (Fig. S4, D) in order to exclude stromal influences. We found that mRNA levels of hOGG1 and POLB were significantly increased in tumor cells as compared to the healthy match, and their expression has been also correlated to the expression of two tumor hypoxia marker genes – DEC1 and CAIX (Zhang and Li, 2007) (Fig. 4, A). At the same time, protein levels of all studied BER genes and both tumor hypoxia markers were significantly increased in NHL IIB cells (Fig. 4, B and C). These observations were cell type independent and a similar expression pattern was found also in three matching biopsies of another canine tumor, namely malignant fibrotic liposarcoma (FLS) with comparable staging (Fig. S4, A-C). Surprisingly, in spite of a low population number, the frequency of tumor samples with overexpressed BER genes was as high as 100%, in contrast to the previous reports showing the average frequency at 30%. Using an *in vitro* BER assay we also detected elevated BER activity in tumors, both for MUTYH and hOGG1 initiated repair, as seen from over 8-fold increased incorporation of [α-P^{32}] dCTP on S1, and [α-P^{32}] dGTP on S2, respectively (Fig. 4, D). Next, we revived NHL IIB samples to obtain B-cells in culture and evaluated their survival potential by CFA. Both revived tumor B-cells and non-transformed splenic lymphocytes were able to tolerate oxidative stress upon re-oxygenation, as shown by increased number of colonies formed following treatment with DNA damaging agents (Fig. 4, E). Moreover, survival of re-oxygenated tumor cells was even higher than cells derived

from matching healthy tissue exposed to hypoxia. Similarly to primary cells, both healthy tissue and tumor derived B-lymphocytes, required unperturbed hOGG1 and POLB function in order to maintain the cell protective effects (Fig. 4, F-G), suggesting a similar mechanism of BER activation to be used also by tumors to tolerate excessive stress and to contribute to treatment resistance.

Discussion

O_2 availability and consumption have long been expected to be a driving force to regulate the expression and function of oxidative DNA repair genes. The rationale behind this is based on data generated in our and other laboratories on the function of BER proteins under oxidative stress conditions. Oxidative stress has been associated with increased oxidative damage to DNA and elevated expression of BER genes (van Loon and Hubscher, 2009). As a byproduct of O_2 reduction in mitochondria, ROS production has also been reported to be in a linear dependence to O_2 utilization and the metabolic activity of a cell (Dirmeier et al., 2004). As a consequence, a linear relationship between ROS formation and expression of BER genes had also been expected since oxidative lesions are direct substrates of core genes in the BER pathway. Based on these data we initially hypothesized that an increase in tissue O_2 partial pressure should lead to increased formation of ROS, cause additional oxidative DNA damage and finally would elevate the expression of BER genes in order to repair the increased oxidative lesion burden. On the contrary, an O_2 shortage should lead to reduced expression of BER genes due to basal or even lowered ROS formation and thus oxidative DNA damage.

To our surprise, the expression of the two core BER enzymes - hOGG1 and DNA pol β gene was simultaneously and significantly up-regulated under hypoxia and, even more remarkable, - maintained for 120 minutes upon re-oxygenation. Acting in short-patch BER hOGG1 and DNA pol β control the levels of 8-oxo-G in the cell (Frosina et al., 1996). Their activation leads to elevated DNA repair capacity, unnecessary under hypoxia, especially from the point of limited energy resources and low level of oxidative DNA damage, but is required later as demonstrated, to exhibit a protective effect against prospective oxidative stress caused by re-oxygenation. This mirrors a situation that often occurs in different tissues in the body as well as during embryonic development. At the same time, the expression of Ape-1, or MUTYH, which together with DNA pol λ forms back up loop for error-free 8-oxo-G bypass in replicating cells (van Loon and Hubscher, 2009), remains unaffected. This finding is supportive for their "guardian mission" in oxidative DNA repair, as perturbation in either expression or activity of these genes leads to multiple pathological conditions (Sampson et al., 2005) (Ohba et al., 2009). The difference between our current and previous findings consists in *how* oxidative stress was induced. In the present study we focused on physiological induction of oxidative stress by hypoxic modulation of ROS production. In most previous reports chemical oxidants, like H_2O_2, or other drugs, e.g. menadion, were used to cause cellular stress. In our opinion, this explains not only the least pronounced changes in 8-oxo-G induction under oxidative stress (e.g. as compared to H_2O_2 treatment), but also previously unreported alterations of expression pattern of BER genes observed under physiological conditions.

Uncoupling expression of BER genes from the rate of ROS production or content of oxidative lesions in the cell is another novel finding of our study. Expression of BER genes

is expected to correlate with the amount of 8-oxo-G in cellular DNA since increase in 8-oxo-G content requires to be handled by an additional quantity of BER proteins. Technical limitations did not allow us to analyze all BER genes, but only HIF-1 dependent members, the expression of which was increased after treatment with DMOG that interferes with HIF-1α stability without affecting 8-oxo-G content in DNA. The HIF-1α pathway has been reported earlier to regulate the expression of DNA pol ι, a member of Y family of DNA pols and being another translesion synthesis (TLS) enzyme (Ito et al., 2006). TLS by DNA pol ι is error-prone thus its involvement in BER is observed mainly under pathological conditions, such as in cancer, where the functions of the core BER genes are often impaired (Lange et al., 2011). The HIF family of transcription factors is known to control many genes involved in adaptation to O_2 (Wenger et al., 2005). The list of its downstream targets is still continuously growing. Our work includes now also DNA pol β and hOGG1 as downstream targets of HIF. Interestingly, the hypoxia-inducible DNA pol λ, the other of two (along to DNA pol β) X family members, is a downstream target of the mTOR kinase, the function of which is linked to active cell growth, autophagy as well as to several pathologies. These events all involve oxidative stress (Sengupta et al., 2010). Distinct pathways controlling key BER DNA pols might be advantageous when extended BER function is needed. Along this line, we note that up-regulation of BER genes by both of these mechanisms resulted in elevated repair capacity of a cell. Although down-regulation of hOGG1 and DNA pol β by specific siRNA supported for direct involvement of absolute protein levels, it still remains unclear whether enzymatic activities of these proteins are also needs to be modified to exhibit cell protective effect since our recent developments demonstrate a critical role of post-translational modifications for the function of other core BER proteins, MUTYH and DNA pol λ (Markkanen et al., 2012).

The data presented in this work allows us to explain not only why the expression of BER genes is activated under non-favorable conditions, but also the possible benefits and biological consequences (Figure 5). Large hypoxic areas characterize the vast majority of tumors in mammals (Lehmann et al., 2009) (Matsumoto et al., 2010) and the severity of hypoxia predicts resistance to chemo- and radiation therapy (Zhou et al., 2006). Along this line we decided to test our finding, using dogs, bearing spontaneously occurring tumors, as a model that closely represents tumor initiation and progress in humans (Khanna et al., 2006). We detected several tumor types with over-expression of both hypoxia markers and BER genes. We therefore assume that activated BER can contribute to survival of hypoxic cells. However, treated with multiple DNA damaging agents, including MMS or γ-irradiation, both primary or revived hypoxic tumor cells were not able to tolerate DNA damage. In contrast to the situation when damaging agents were applied at later time point during re-oxygenation and were expected to cause even more cellular stress, they were tolerated to a large extent, including γ-irradiation. As it has been reported for some cases of drug resistance (Valko et al., 2006), in our study, increased protein levels or elevated enzymatic activity of detoxifying enzymes was not detected. Therefore activation of BER in this case is the only mechanism that allows for excessive oxidative stress tolerance, which in a way is the same for primary cells or tumor cells from dogs, points for an universality of our findings. Furthermore, activity of BER in either NHL IIB samples, or FLS sample was elevated and dependent on the level of BER genes expression thus making hyperfunction of BER to be one of the factors in tumor treatment resistance. Our data are independently supported in a study where

human colon cancer biopsies were compared to surrounding healthy tissues (Kirkali et al., 2011).

The forces of BER activation *in vivo* are still awaiting further elucidation. In this direction, multiple reports highlight the importance of tissue O_2 dynamics. Impressively, the dynamics of perfusion and O_2 persistence in the tissue ranges from just 20 milliseconds, in mouse brain under stress (Shen et al., 2009), to many days in a developing human embryo (Dunwoodie, 2009). Tumor tissue known for its chaotic vascularization is an excellent example. A high rate of cell proliferation in addition to dysfunctional perfusion constantly induce intermittent hypoxia in the growing tumor mass (Matsumoto et al., 2010), thereby activating BER genes. Finally, the littoral crab (*Carcinus maenas*), which can dive up to 40 meters deep and returns back to sea level, is repeatedly exposed to O_2 concentrations between 0.01% and 20% for at least 30 times a day known for its high stress tolerance (Taylor, 1976). Conclusively, the mentioned processes would all be deleterious for living cells and organisms due to increased ROS production and high rate of oxidative DNA damage unless a general mechanism to combat that danger would exist as an "insurance" and can be adjusted accordingly to potential needs to sudden changes in the environment or in the body. Such changes in the body can be physiological as well as pathological (e.g. cancer microenvironment).

Experimental procedures

Chemicals

Deoxyribonucleoside triphphosphates were purchased from Sigma. Labeled [α-^{32}P] dGTP and [α-^{32}P] dCTP were purchased from Hartmann Analytic (Germany). All other reagents were of analytic grade and purchased from Fluka, Sigma or Merck. Microsynth synthesized the oligonucleotides, and 3'-biotinylated 58-mer were from Purimex. Streptavidin-coupled magnetic beads (Cat. #112.05D) were purchased from Invitrogen Dynal AG (Oslo, Norway).

Cell cultures

All cells were maintained under 95% air/5% CO_2 in DMEM supplemented with 15% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). For hypoxia and subsequent re-oxygenation cells were grown and passaged in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK) using reagents pre-equilibrated to the oxygen concentration in the glove box. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Reverse transcription quantitative RT-PCR

Total RNA was extracted using TRIzol® reagent (Cat. #15596-026, Invitrogen, CA, USA) accordingly to the manufacturer's instructions. After treatment with DNase I (Cat. #776785, Roche Diagnostics, Mannheim, Germany), fraction of messenger RNA (mRNA) was precipitated by addition of LiCl to 2.5 M at -20 ^{0}C and 3 µg of mRNA was used in Superscript III (Cat. #18080-044, Invitrogen, Basel, Switzerland) reverse transcription (RT) accordingly to the producer's protocol. Messenger RNA levels were quantified with 5% of the diluted complementary DNA reaction mix by quantitative polymerase chain reaction (qPCR) using gene specific primers (Table S1) and SensiMixPlus SYBR kit (Cat. #QT-605-02, Quantace Ltd., UK) in combination with a Rotor Gene RG-3000A light cycler (Corbett Research, QIAGEN, Germany). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein L28 (RPL28) mRNA was determined and data expressed as ratios relative to L28 levels. Melting point analyses of amplified PCR products were performed after each run to verify specific amplification.

Protein extractions and immunoblot analyses

Cells were washed twice and scraped into ice-cold PBS. For BER and enzymatic assays cellular proteins were extracted with a high-salt buffer containing 0.5% NP-40 and protein concentrations were determined by the Bradford method as described before (Wimmer et al., 2008). Alternatively, total cell lysates were prepared to analyze protein levels. Therefore, cells were collected in ice-cold PBS, lysed with 4% sodium dodecylsulfate (SDS), 120 mM Tris-HCl (pH 6.8) and 20% glycerol, followed by sonification and boiling for 5 min. In this case, protein concentrations were determined using the Lowry assay. Protein (50-80 µg) was separated by SDS–polyacrylamide gel electrophoresis, electro-transferred onto polyvinylidene difluoride membranes and probed with primary antibodies derived against proteins of interest (Table S2). Fluorescence labeled secondary antibodies and Odyssey Infrared Imaging System (LI-COR GmbH, Germany) were used to detect and analyze signals.

Base Excision Repair (BER) Assays

Repair assays were performed in a reaction mixture (20 µl) containing 50 mM HEPES (pH 7.4), 20 mM KCl, 2 mM DTT, 10 mM $MgCl_2$, 2 mM ATP, 2 µM 5′[α-^{32}P] dGTP/dATP/dCTP (3000 Ci/mmol), and 300 pmol of either substrate DNA. The reactions were initiated by adding 10 µg of control or sample whole cell extract, incubated at 37°C for 30 min and then stopped with 5 µl of 0.25 M EDTA.

Reactive Oxygen Species (ROS) Detection

Intracellular ROS production was determined by flow cytometry as a rate of DCFH oxidation according to Mahfouz et al. with modifications (Mahfouz et al., 2009). Cells were washed twice with PBS and incubated with 100 µM of carboxy-H_2DCF-DA (Cat. #C-400, Molecular Probes, Invitrogen, Basel, Switzerland) at 37°C for 30 min in culture medium supplemented with 1% (v/v) FCS, which was substituted for PBS on completion. The DCF fluorescence was evaluated between 500 and 530 nm using a flow cytometer equipped with a 488-nm argon laser as a light source (CyAn ADP Analyzer, Beckman Coulter International S.A., Switzerland). Data were expressed as the percentage of fluorescent cells.

Flow Cytometry

All fluorescence signals of labeled cells were analyzed by the flow cytometer CyAn ADP Analyzer (Beckman Coulter International S.A., Switzerland). A minimum of 100,000 cells were examined for each assay at a flow rate of <100 cells/second. The cell population was gated using 90R° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. The percentage of positive cells and the mean fluorescence were calculated on a 1023-channel scale and analyzed using the Summit Software (Beckman Coulter International S.A., Switzerland).

Immunofluorescence

10^4 cells were seeded on 60 mm dishes containing 10 mm glass cover slips. Following treatment cells were rinsed with PBS and fixed with 100% methanol at -30°C for 10 min. After blocking with 10% fetal calf serum for 1 hour, cover slips were moved to 24-well plate, the primary antibodies (Table S2) were allowed to bind for 2 hours and immune complexes were detected using secondary anti-rabbit Alexa Fluor 488 (Cat. #A-11008)/Texas Red (Cat. #T-2767) - and anti-mouse Alexa Fluor 488 (Cat. #A-11001)/Texas Red (Cat. #T-862) - conjugates, respectively (Molecular Probes, Invitrogen, Basel, Switzerland). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Glass cover slips were dried for 30 min and mounted in DPX (Cat. #44587, Sigma, Buchs, Switzerland) in the dark. Epifluorescence was analyzed using an Olympus BX51 microscopy system (Olympus, Volketswil, Switzerland) and images were captured with fixed exposure times.

Colony formation assays

$1*10^5$ cells were cultured on 100 mm tissue culture plates for 24 hours exposed to different oxygen conditions (see Cell cultures) for 16 hours and subsequently treated with 1 mM H_2O_2 (Cat. #H1009, Sigma, Buchs, Switzerland) for the last 2 hours of incubation, or during re-oxygenation. Cells were then trypsinized, counted and 10^3 cells seeded on 60 mm culture plates and grown for an additional 7 days. Following fixation with 6% glutaraldehyde in

phosphate-buffered saline (PBS), the colonies were stained with crystal violet, counted and the numbers normalized to the untreated control groups.

RNA interference
MRC5 cells were plated at a density of $3x10^5$ cells per 60 mm culture plate and allowed to adhere overnight and then transfected with 100 pmol of small interfering RNA (siRNA) targeting POLL (Qiagen ID 9), POLB (Qiagen ID 12), MUTYH (Qiagen ID 11) or hOGG1/2 (Cat. #SC-43983, Santa Cruz Biotechnology, CA, USA) genes using Lipofectamine RNAiMAX (Cat. #13778-150, Invitrogen, Basel, Switzerland) according to the manufacturer's protocol. Following 48 hours, cells were processed as required for subsequent procedures.

HIF-α knockdown cells and lentiviral infections
HEK293 shHIF1α clones were generated by transfection with a pLKO.1-puro vector expressing U6 promoter driven shRNA-targeting nucleotides 1168-1188 of human HIF-1α (NM_001530.x-1048s1c1, Sigma). Cell clones were derived by puromycin selection (2 μg/ml) and ring cloning. Lentiviral expression vectors encoding three shRNA sequences targeting human HIF-2α at nucleotides 878-898 (#1, NM_001430.x-517s1c1), 927-947 (#5, NM_001430.x-566s1c1) and 2055-2075 (#4, NM_001430.x-1694s1c1) in a pLKO.1-puro plasmid were purchased from Sigma. Viral particles were produced in HEK293T cells by co-transfection of the respective transfer vector (3 μg) with the packaging plasmids pLP1 (4.2 μg), pLP2 (2 μg) and pVSV-G (2.8 μg, all from Invitrogen) using PEI transfection as described before (Stiehl et al., 2006) HEK293 cells were infected with lentiviral-pseudo typed particles and cell pools were derived by puromycin (2 μg/ml) selection.

Statistical Analysis
Statistical analyses were carried out by one-way ANOVA with post-Tukey multiple comparison test using the XLSTAT®-Pro 2011 statistical analysis add-on for Microsoft® Excel® (SAS Institute Inc., NC, USA). All tests were two-sided with statistical significance set at $p < 0.05$(**).

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Figure legends

Figure 1. Hypoxia activates BER via multiple pathways. (A) Expression of core BER and hypoxia mRNAs in MRC5 cells analyzed by RT-qPCR. Shown are mean values +/- SEMs of five independent experiments. (B) Expression of core BER and hypoxia proteins in MRC5 cells analyzed by immunoblotting. Median intensity of protein bands was expressed relative to β-tubulin control. Shown are mean values +/- SEMs of three independent experiments. (C). Quantification of B. (D) Expression of 4 core BER and 3 hypoxia-inducible mRNAs in parental (wild type) and HIF-1α/HIF-2α shRNA transfected HEK293 cells analyzed by RT-qPCR. The cells were analyzed at 20% and 0.2% O_2, respectively. (E) Expression of POLB, hOGG1 and HIF-1α proteins in parental (wild type) and HIF-1α/HIF-2α shRNA transfected HEK293 cells under hypoxia (0.2% $O_{2)}$ analyzed by immunoblotting. (F) Oligonucleotide constructs (substrates) used for the *in vitro* BER assay. (G) Schematic representation of *in vitro* BER assay experiments. For further explanation see text. (H) *In vitro* BER assay of MRC5 cells cultured for 16 hours at the indicated oxygen atmosphere. Shown are mean values of incorporation of radioactively labeled deoxynucleoside triphosphates subtracted to control +/- SEMs of five independent experiments. (I) *In vitro* BER assay of parental (wild type) and HIF-1α/HIF-2α shRNA transfected HEK293 cells cultured for 16 hours at respective oxygen atmosphere. Shown are mean values of incorporation of radioactively labeled deoxynucleoside triphosphates subtracted to control +/- SEMs of three experiments.

Figure S1. Hypoxia activates BER via multiple pathways. (A) Expression of POLB, hOGG1, MUTYH and GLUT1 mRNAs in MRC5 cells cultured for 16 hours under the indicated oxygen atmosphere analyzed by RT-qPCR. Shown are mean values +/- SEMs of five independent experiments. (B) mRNA levels of the angiogenesis factors VEGF, bFGF, TGF-β, and the hypoxia marker GLUT1 in MRC5 cells cultured for 16 hours under the indicated oxygen atmosphere analyzed by RT-qPCR. Shown are mean values +/- SEMs of three independent experiments. (C) Schematic representation of *in silico* predicted hypoxia response elements (HREs). (D) HREs identified in the promoter regions of core BER and control gene sets. TSS: transcription start site; numbers show the position of the first nucleotide in the HRE motif relative to TSS. (E) Reporter gene assay of *in silico* identified HREs in POLB and hOGG1promoter regions. WT: native HRE sequence, mut: HRE sequence disrupted for HIF binding site. R.L.U., relative light units. (F) Expression of POLL and MUTYH mRNAs in MRC5 cells cultured for 16 hours under the indicated oxygen atmosphere and treated with 200 nM rapamycin for the last 2 hours analyzed by RT-qPCR. Shown are mean values +/- SEMs of two experiments. (G) Expression of POLL and MUTYH proteins in MRC5 cells cultured for 16 hours under the indicated oxygen atmosphere and treated with 200 nM rapamycin for the last 2 hours of incubation analyzed by immunoblotting. P70S6K expression controlled mTOR activity and β-tubulin was used as a loading control. (H) Expression of POLL and MUTYH proteins in MRC5 cells plated at different cell density and cultured for 16 hours under the indicated oxygen atmosphere analyzed by immunoblotting.

Figure 2. The activation of BER genes relies on hypoxia but not on the extent of ROS production or on the accumulation of oxidative DNA lesions. (A) Rates of intracellular ROS production in MRC5 cells cultured for 16 hours under the indicated oxygen

atmosphere. Percentage of DCF positive cells is indicated. (B) Area under the curve (AUC) plotted from three independent experiments described in A. (C) 8-oxo-G accumulation analyzed by immunofluorescence with a specific antibody. (D) Mean fluorescence intensity of 8-oxo-G calculated for 200 cells +/- SEMs in C. (E) Expression of core BER and hypoxia mRNAs in MRC5 cells cultured for 16 hours under the indicated oxygen atmosphere and treated with 1 mM DMOG analyzed by RT-qPCR. Shown are mean values +/- SEMs of five independent experiments. (F) Expression of core BER and hypoxia proteins in MRC5 cells treated with 1 mM DMOG analyzed by immunoblotting. Median intensity of protein bands was expressed relative to the β-tubulin control. Shown are mean values +/- SEMs of three independent experiments. (G) Quantification of F. (H) Accumulation of 8-oxo-G and expression of HIF-1α protein analyzed by immunofluorescence in MRC5 cells cultured for 16 hours at 20% O_2 and treated with 1 mM DMOG. (I) Mean fluorescence intensity calculated for 200 cells +/- SEMs is shown. (J) Expression of POLB and hOGG1 proteins analyzed by immunofluorescence in MRC5 cells cultured for 16 hours at 20% O_2 and treated with 1 mM DMOG. (K) Mean fluorescence intensity calculated for 200 cells +/- SEMs is shown.

Figure S2. The activation of BER genes relies on hypoxia but not on the extent of ROS production or on the accumulation of oxidative DNA lesions. (A) Rates of intracellular ROS production in MRC5 cells cultured for 16 hours at 5% oxygen and treated with 1 mM H_2O_2, PBS or 1 mM DTT for the last two hours of incubation. Percentage of DCF positive cells is indicated. (B) 8-oxo-G accumulation analyzed by flow cytometry with a specific antibody. Percentage of positive cells is indicated. (C) Detection of 8-oxo-G in DNA of MRC5 cells by the combined hybridization-immunoblot technique. (D) Co-staining of 8-oxo-G and MitroTracker® Green, a mitochondria specific probe. (E-F) Expression of POLL (E) and CAIX (F) proteins analyzed by immunofluorescence in MRC5 cells cultured for 16 hours at 20% O_2 and treated with 1 mM DMOG.

Figure 3. Activation of BER leads to an increased cell survival in response to the oxidative stress. (A) Colony formation assay of MRC5 cells treated with 1 mM H_2O_2 during 120 min of re-oxygenation. Shown are mean values +/- SEMs of three experiments. (B) Silencing of core BER genes under hypoxia. MRC5 cells were transfected with respective siRNA 48 hours prior to the differential oxygenation. (C-F) Colony forming assay of MRC5 cells with siRNA silenced BER genes (C: POLL, D: POLB, E: OGG1, F: MUTYH) treated with 1 mM H_2O_2 during 120 min of re-oxygenation. Shown are mean values +/- SEMs of three experiments.

Figure S3. Activation of BER leads to an increased cell survival in response to the oxidative stress. (A) Colony formation assay of MRC5 cells treated with 1 mM H_2O_2 during last 2 hours of hypoxia. (B) Colony formation assay of MRC5 cells treated with 100 nM MMS during 120 minutes of re-oxygenation. (C) Colony formation assay of MRC5 cells treated with 3 Gy of γ-irradiation during 120 minutes of re-oxygenation. Shown are mean values +/- SEMs of three independent experiments (A, B and C) (D) MTT assay of MRC5 cells treated with 1 mM H_2O_2, 100 nM MMS and 3 Gy of γ-irradiation during 120 minutes of re-oxygenation. Cells were allowed to recover for 24 hours after treatment. Mean values +/- SEMs of five independent experiments are shown. (E) Expression of catalase and GSH

peroxidase proteins in MRC5 cells analyzed by immunoblotting. (F) Activity of catalase in MRC5 cells treated with 1 mM H_2O_2 for 120 minutes of re-oxygenation analyzed in an *in vitro* assay using whole cell extracts. Arrowed labels represent treatment with 100 nM MMS. (G-I) Activity of GSH peroxidase in untreated (G), or treated with 1 mM H_2O_2 (H) or 100 nM MMS (I) MRC5 cells during 120 minutes of re-oxygenation analyzed by an in vitro assay using whole cell protein extracts. Mean values +/- SEMs of a five independent experiments are shown (for F-I).

Figure 4. Activated BER genes can be identified in dog tumors. (A) mRNA expression levels of core BER and hypoxia-inducible genes in canine NHL samples (n=8) analyzed by RT-qPCR. Shown are mean values +/- SEMs. (B) Expression of core BER and hypoxia proteins in canine NHL samples (n=8) analyzed by immunoblotting. Median intensity of protein bands was expressed relative to β-tubulin control. Shown are mean values +/- SEMs. (C) Quantification of B. (D) *In vitro* BER assay of canine NHL samples (n=8). Shown are mean values of incorporation of radioactively labeled deoxyribonucleoside triphosphates subtracted to control +/- SEMs. (E) Colony formation assay of revived tumor and matching non-transformed B-lymphocytes obtained from NHL samples (n=8) treated with 1 mM H_2O_2 during 120 minutes of re-oxygenation. Shown are mean values +/- SEMs. (F) Silencing of core BER genes in revived tumor B-lymphocytes under hypoxia. Cells were transfected with respective siRNA 48 hours prior to the differential oxygenation. (G-H) Colony forming assay of revived tumor and matching non-transformed B-lymphocytes obtained from NHL samples (n=8) with siRNA silenced BER genes (G: POLB, H: OGG1) treated with 1 mM H_2O_2 during 120 minutes of re-oxygenation. Shown are mean values +/- SEMs.

Figure S4. Activated BER genes can be identified in dog tumors. (A) Expression of core BER and hypoxia mRNAs in canine FLS samples (n=3) analyzed by RT-qPCR. Shown are mean values +/- SEMs. (B) Expression of core BER and hypoxia proteins in canine FLS samples (n=3) analyzed by immunoblotting. Median intensity of protein bands was expressed relative to β-tubulin control. Shown are mean values +/- SEMs. (C) Quantification of B. (D) Fractionation of NHL and FLS tumor samples by flow cytometry. Percentage of positive cells is indicated, +/- SEM.

Figure 5. Model for regulation of BER proteins leading to subsequent increased survival upon hyperoxia. For details see text.

Figures and Tables

Figure 1

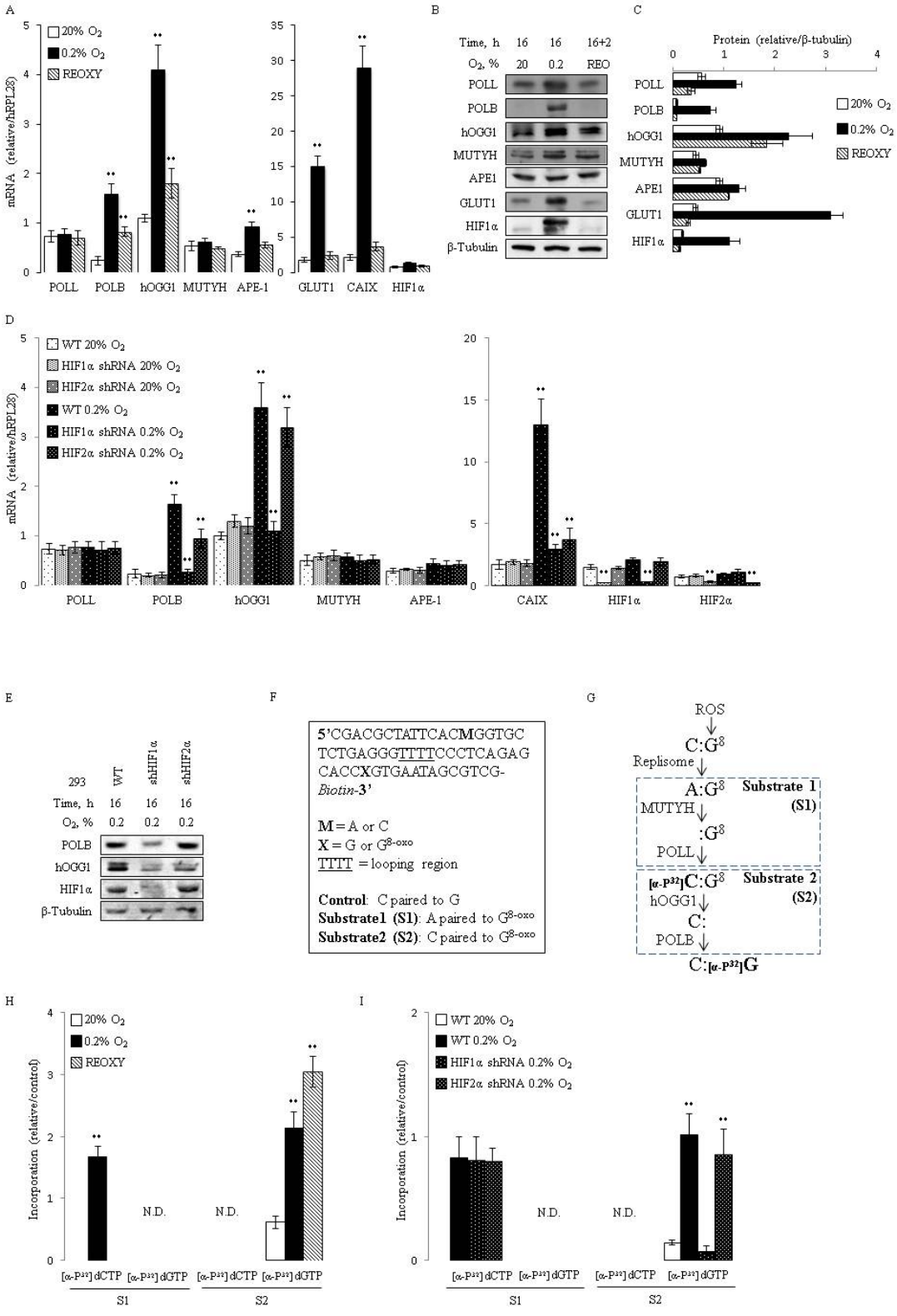
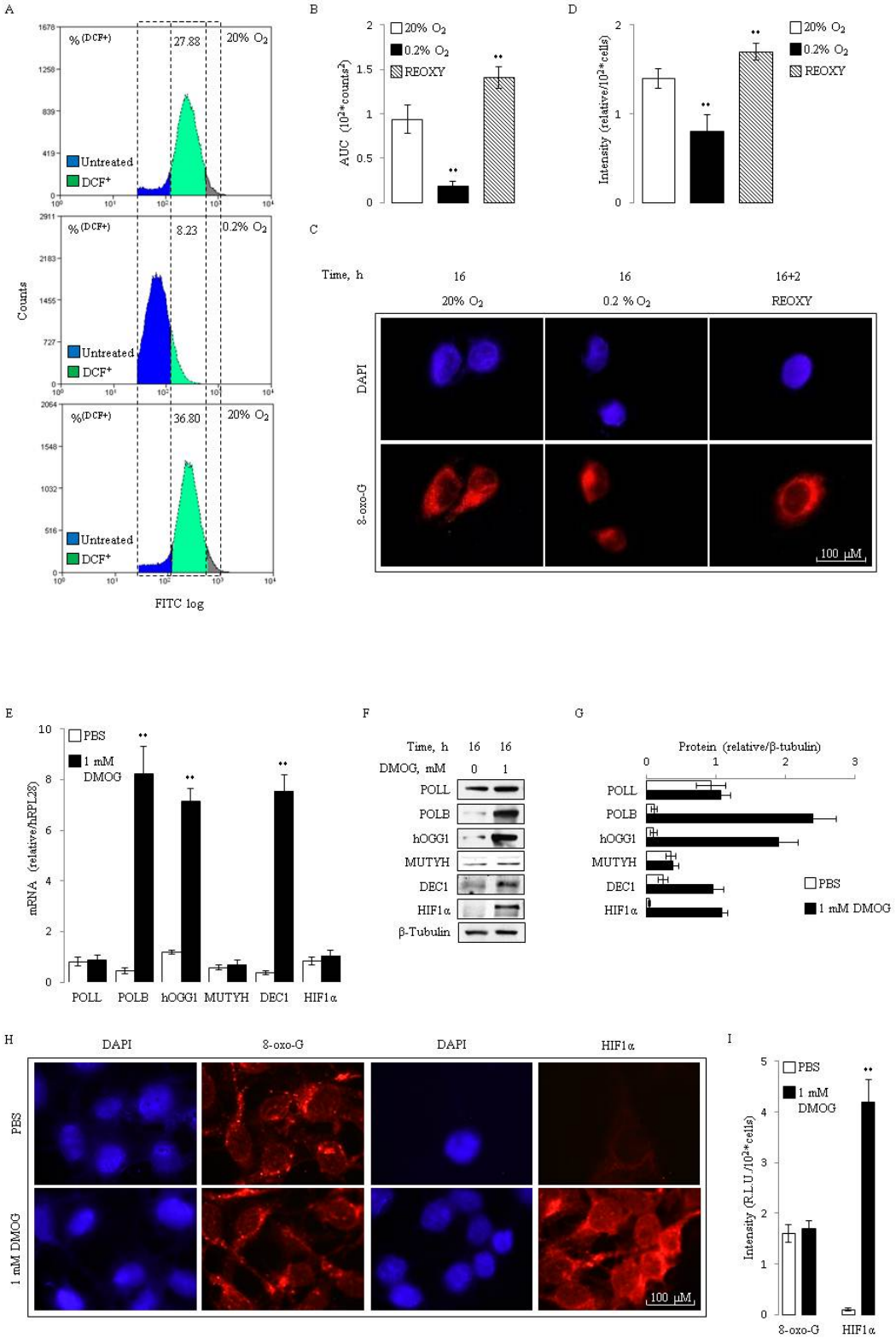
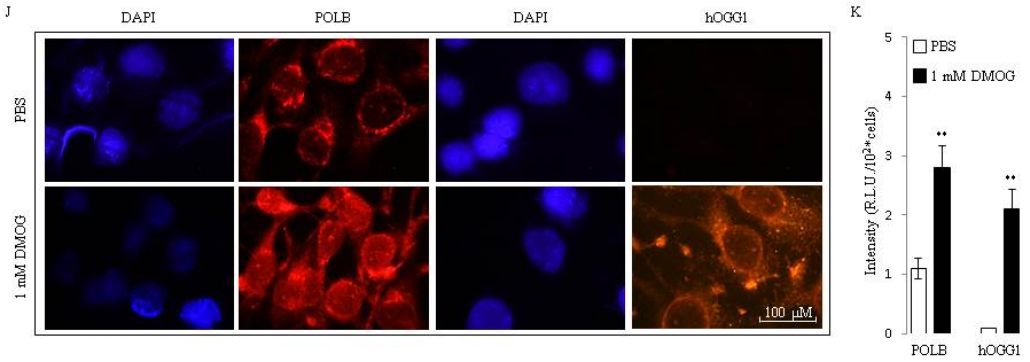


Figure 2


A
% (DCF+)
27.88
20% O2
Untreated
DCF+
8.23
0.2% O2
36.80
Counts
FITC log
B
20% O2
0.2% O2
REOXY
AUC (10²*counts²)
D
Intensity (relative/10²*cells)
C
Time, h
16
16
16+2
20% O2
0.2 % O2
REOXY
DAPI
8-oxo-G
100 μM
E
PBS
1 mM DMOG
mRNA (relative/hRPL28)
POLL
POLB
hOGG1
MUTYH
DEC1
HIF1α
F
Time, h
DMOG, mM
β-Tubulin
G
Protein (relative/β-tubulin)
H
DAPI
8-oxo-G
HIF1α
PBS
1 mM DMOG
I
Intensity (R.L.U./10²*cells)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DAPI
POLB
DAPI
hOGG1
PBS
1 mM DMOG
100 μM
K
PBS
1 mM DMOG
Intensity (R.L.U./10^2*cells)
POLB
hOGG1

Figure 3

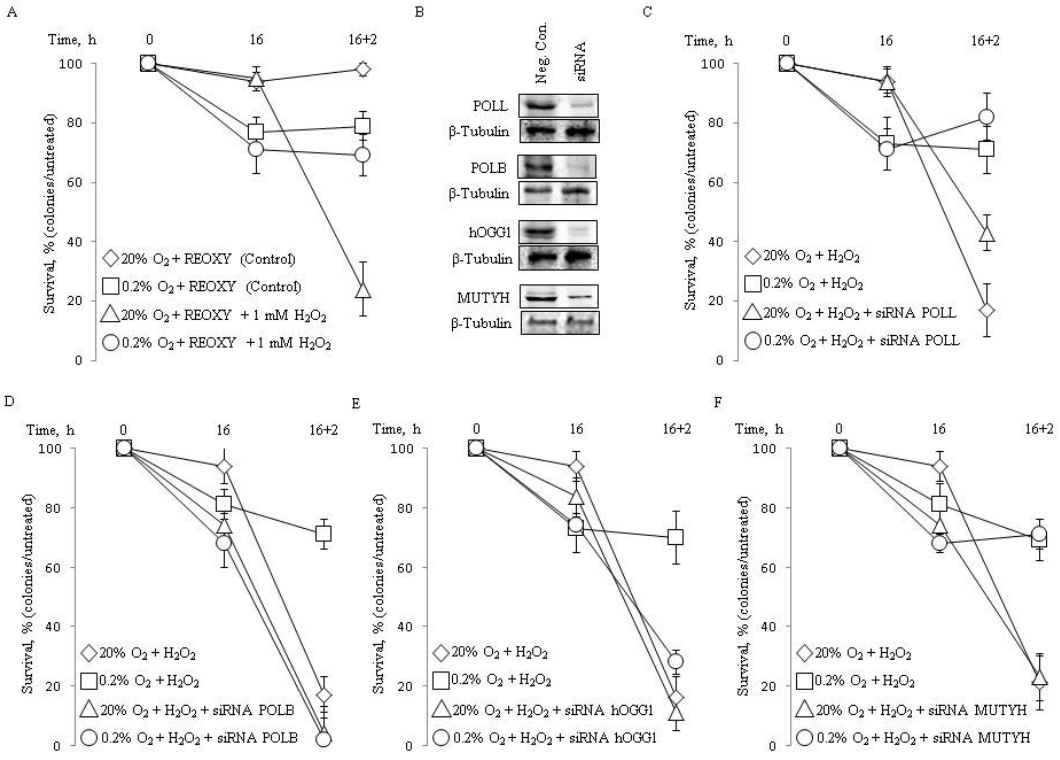
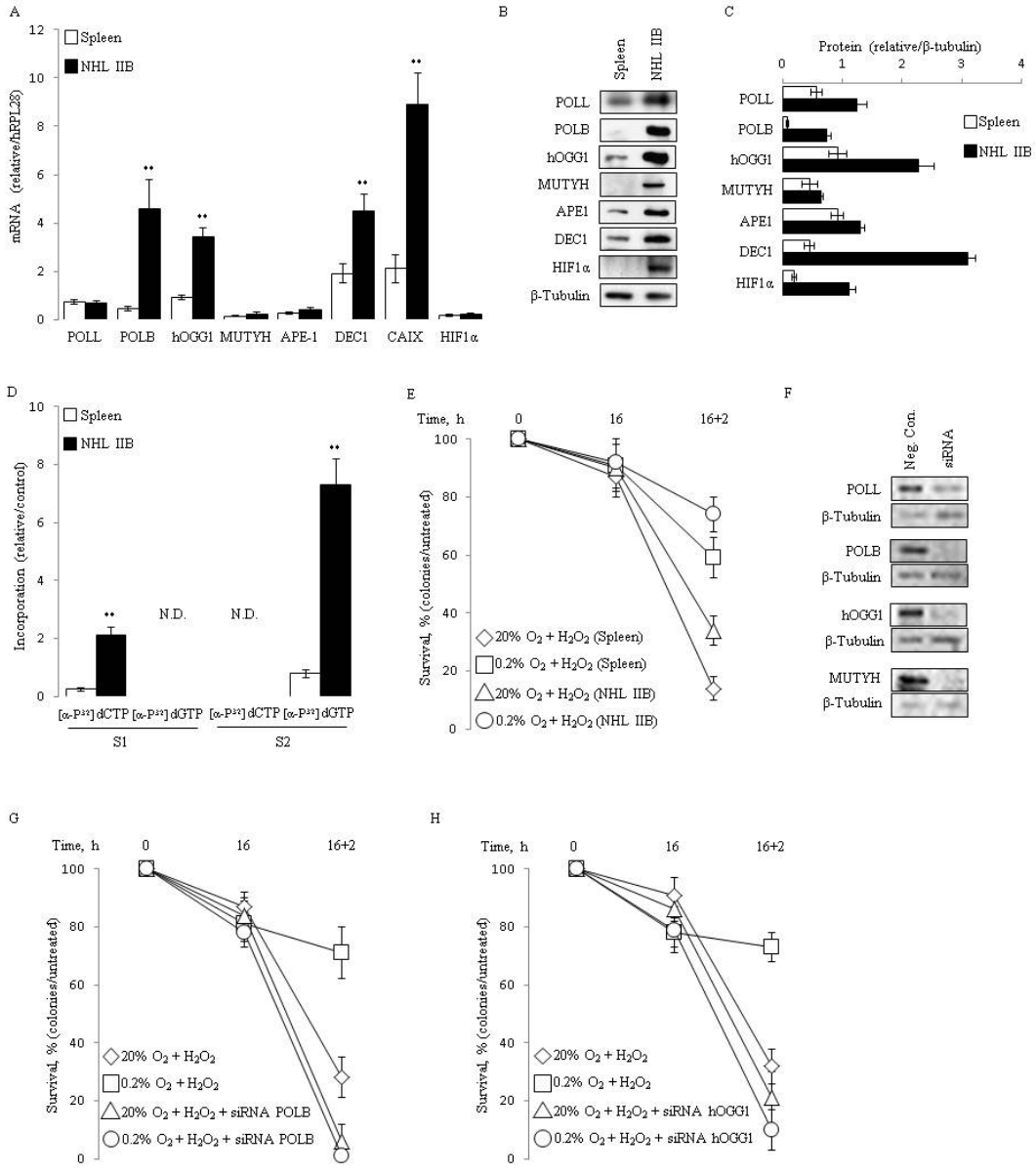

A
Time, h
0
16
16+2
Survival, % (colonies/untreated)
20% O2 + REOXY (Control)
0.2% O2 + REOXY (Control)
20% O2 + REOXY + 1 mM H2O2
0.2% O2 + REOXY + 1 mM H2O2
B
Neg. Con.
siRNA
POLL
β-Tubulin
POLB
β-Tubulin
hOGG1
β-Tubulin
MUTYH
β-Tubulin
C
20% O2 + H2O2
0.2% O2 + H2O2
20% O2 + H2O2 + siRNA POLL
0.2% O2 + H2O2 + siRNA POLL
D
20% O2 + H2O2
0.2% O2 + H2O2
20% O2 + H2O2 + siRNA POLB
0.2% O2 + H2O2 + siRNA POLB
E
20% O2 + H2O2
0.2% O2 + H2O2
20% O2 + H2O2 + siRNA hOGG1
0.2% O2 + H2O2 + siRNA hOGG1
F
20% O2 + H2O2
0.2% O2 + H2O2
20% O2 + H2O2 + siRNA MUTYH
0.2% O2 + H2O2 + siRNA MUTYH

Figure 4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Spleen
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POLB
hOGG1
MUTYH
APE-1
DEC1
CAIX
HIF1α
B
Spleen
NHL IIB
POLL
POLB
hOGG1
MUTYH
APE1
DEC1
HIF1α
β-Tubulin
C
Protein (relative/β-tubulin)
Spleen
NHL IIB
D
Incorporation (relative/control)
N.D.
[α-P³²] dCTP [α-P³²] dGTP
S1
S2
E
Time, h
Survival, % (colonies/untreated)
20% O2 + H2O2 (Spleen)
0.2% O2 + H2O2 (Spleen)
20% O2 + H2O2 (NHL IIB)
0.2% O2 + H2O2 (NHL IIB)
F
Neg. Con.
siRNA
G
20% O2 + H2O2
0.2% O2 + H2O2
20% O2 + H2O2 + siRNA POLB
0.2% O2 + H2O2 + siRNA POLB
H
20% O2 + H2O2 + siRNA hOGG1
0.2% O2 + H2O2 + siRNA hOGG1

Figure 5

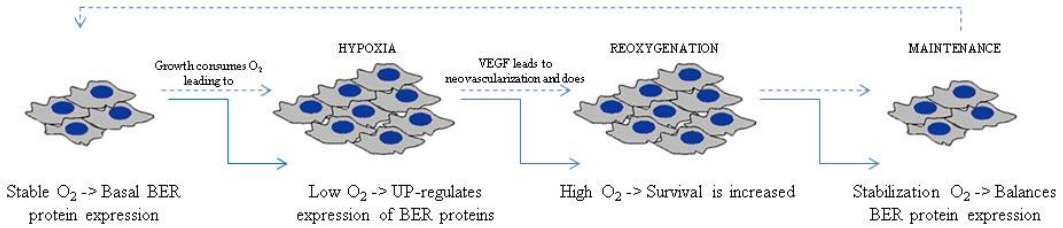

HYPOXIA
REOXYGENATION
MAINTENANCE
Growth consumes O_2 leading to
VEGF leads to neovascularization and does
Stable O_2 -> Basal BER protein expression
Low O_2 -> UP-regulates expression of BER proteins
High O_2 -> Survival is increased
Stabilization O_2 -> Balances BER protein expression

Figure S1

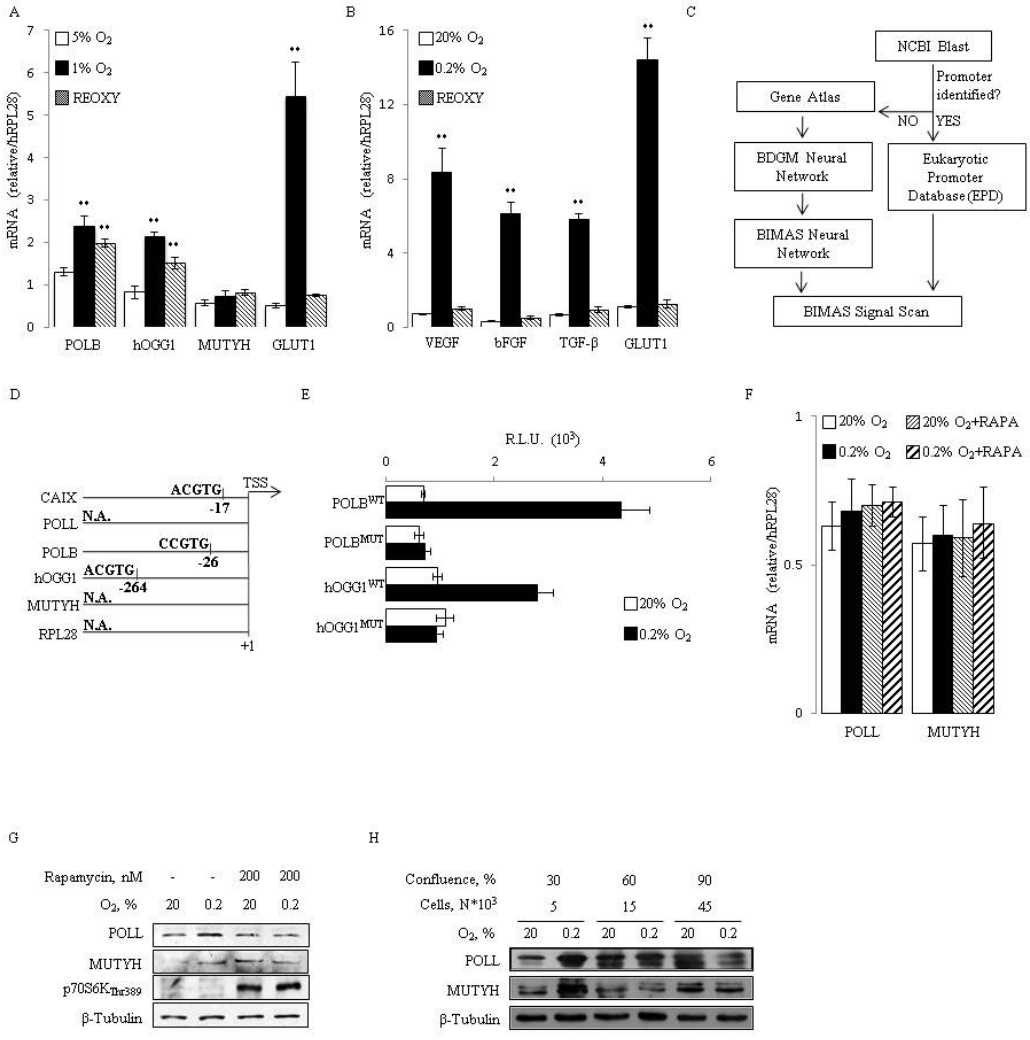
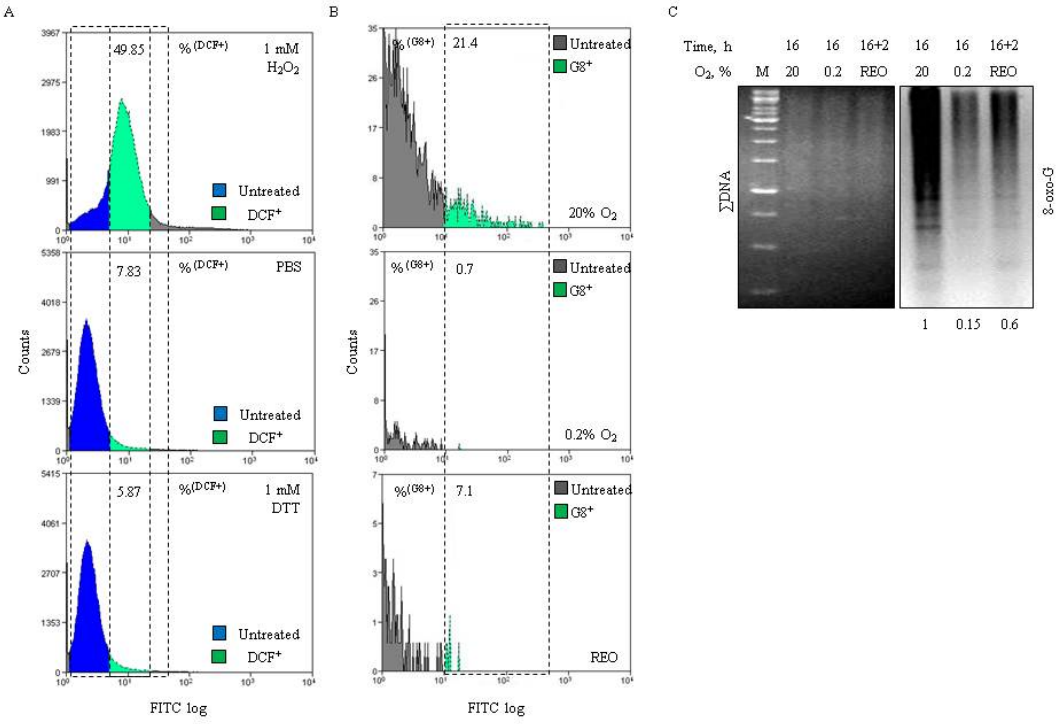
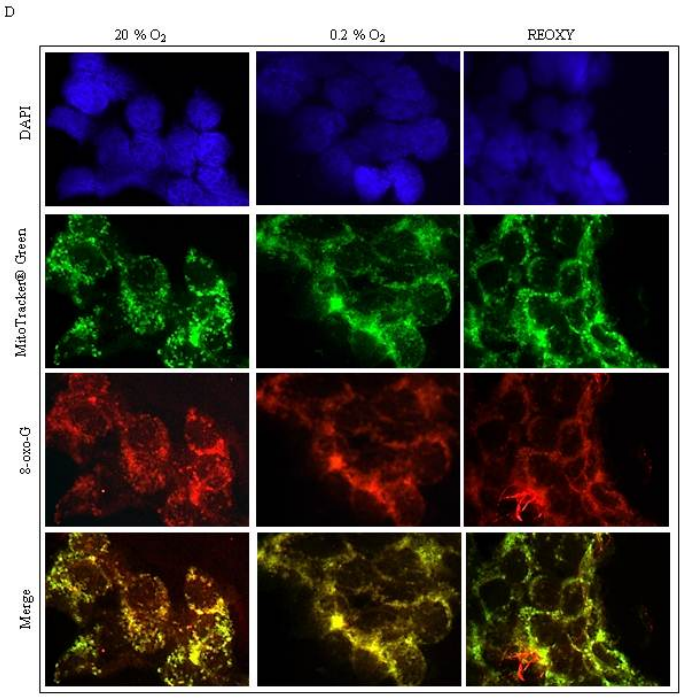

A
5% O2
1% O2
REOXY
mRNA (relative/hRPL28)
POLB
hOGG1
MUTYH
GLUT1
B
20% O2
0.2% O2
REOXY
mRNA (relative/hRPL28)
VEGF
bFGF
TGF-β
GLUT1
C
NCBI Blast
Promoter identified?
Gene Atlas
NO
YES
BDGM Neural Network
Eukaryotic Promoter Database (EPD)
BIMAS Neural Network
BIMAS Signal Scan
D
CAIX
POLL
POLB
hOGG1
MUTYH
RPL28
ACGTG
CCGTG
N.A.
-17
-26
-264
TSS
+1
E
R.L.U. (10³)
POLB^WT
POLB^MUT
hOGG1^WT
hOGG1^MUT
20% O2
0.2% O2
F
20% O2
20% O2+RAPA
0.2% O2
0.2% O2+RAPA
mRNA (relative/hRPL28)
POLL
MUTYH
G
Rapamycin, nM
O2, %
POLL
MUTYH
p70S6K Thr389
β-Tubulin
H
Confluence, %
Cells, N*10³
O2, %
POLL
MUTYH
β-Tubulin

Figure S2


A
B
C
49.85
%(DCF+)
1 mM H2O2
Untreated
DCF+
7.83
%(DCF+)
PBS
5.87
%(DCF+)
1 mM DTT
Counts
FITC log
%(G8+)
21.4
Untreated
G8+
20% O2
0.7
0.2% O2
7.1
REO
Time, h
16
16
16+2
16
16
16+2
O2, %
M
20
0.2
REO
20
0.2
REO
ΣDNA
8-oxo-G
1
0.15
0.6


D
20 % O2
0.2 % O2
REOXY
DAPI
MitoTracker® Green
8-oxo-G
Merge

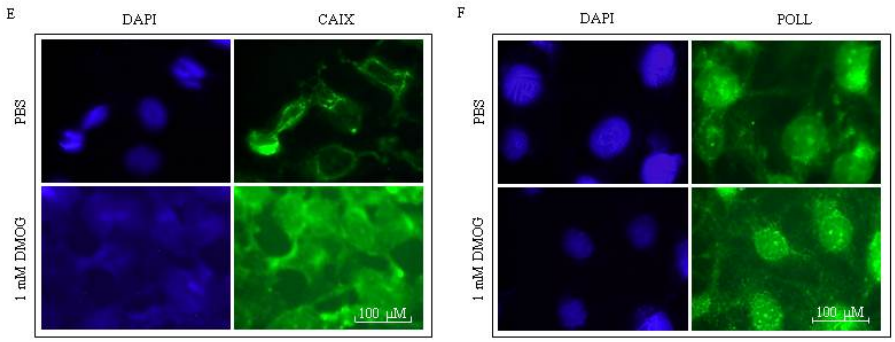

E
DAPI
CAIX
PBS
1 mM DMOG
100 μM
F
DAPI
POLL
PBS
1 mM DMOG
100 μM

Figure S3

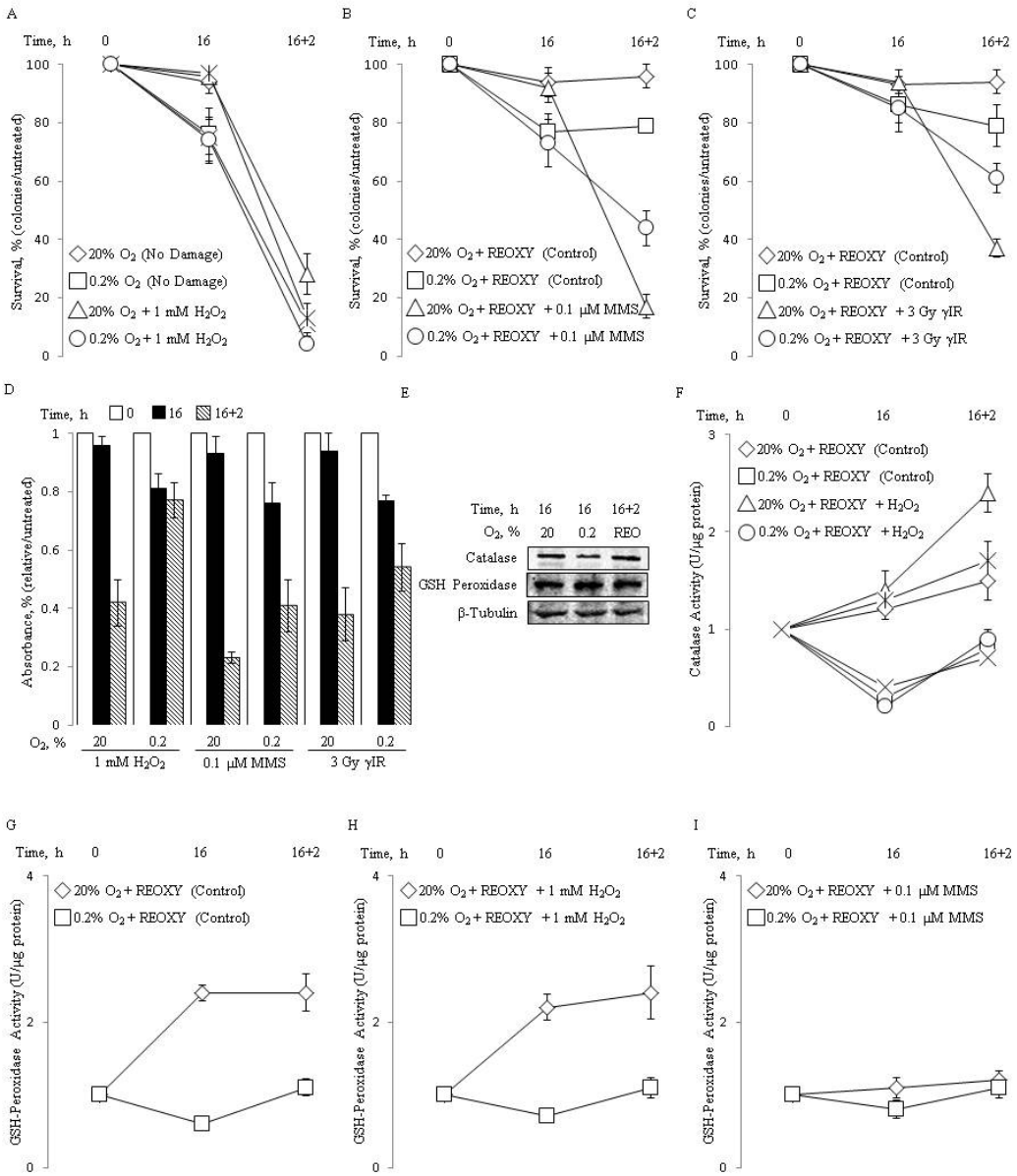
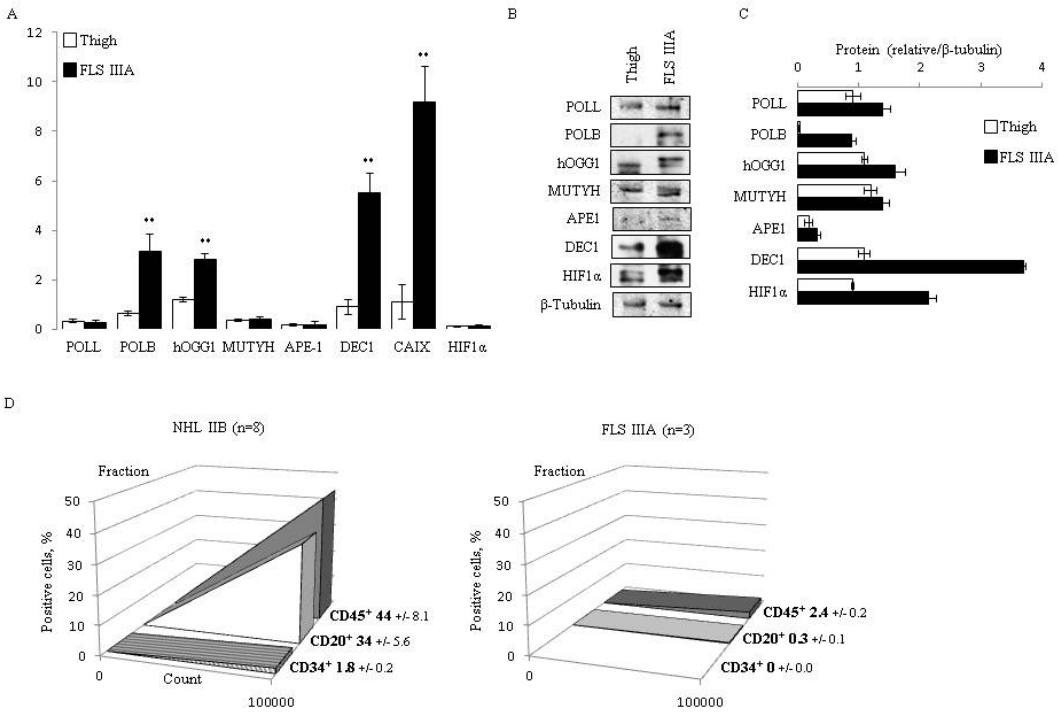

A
Time, h 0 16 16+2
Survival, % (colonies/untreated)
20% O2 (No Damage)
0.2% O2 (No Damage)
20% O2 + 1 mM H2O2
0.2% O2 + 1 mM H2O2
B
Time, h 0 16 16+2
Survival, % (colonies/untreated)
20% O2 + REOXY (Control)
0.2% O2 + REOXY (Control)
20% O2 + REOXY + 0.1 μM MMS
0.2% O2 + REOXY + 0.1 μM MMS
C
Time, h 0 16 16+2
Survival, % (colonies/untreated)
20% O2 + REOXY (Control)
0.2% O2 + REOXY (Control)
20% O2 + REOXY + 3 Gy γIR
0.2% O2 + REOXY + 3 Gy γIR
D
Time, h 0 16 16+2
Absorbance, % (relative/untreated)
O2, % 20 0.2 20 0.2 20 0.2
1 mM H2O2
0.1 μM MMS
3 Gy γIR
E
Time, h 16 16 16+2
O2, % 20 0.2 REO
Catalase
GSH Peroxidase
β-Tubulin
F
Time, h 0 16 16+2
Catalase Activity (U/μg protein)
20% O2 + REOXY (Control)
0.2% O2 + REOXY (Control)
20% O2 + REOXY + H2O2
0.2% O2 + REOXY + H2O2
G
Time, h 0 16 16+2
GSH-Peroxidase Activity (U/μg protein)
20% O2 + REOXY (Control)
0.2% O2 + REOXY (Control)
H
Time, h 0 16 16+2
GSH-Peroxidase Activity (U/μg protein)
20% O2 + REOXY + 1 mM H2O2
0.2% O2 + REOXY + 1 mM H2O2
I
Time, h 0 16 16+2
GSH-Peroxidase Activity (U/μg protein)
20% O2 + REOXY + 0.1 μM MMS
0.2% O2 + REOXY + 0.1 μM MMS

Figure S4


A
Thigh
FLS IIIA
POLL
POLB
hOGG1
MUTYH
APE-1
DEC1
CAIX
HIF1α
B
Thigh
FLS IIIA
POLL
POLB
hOGG1
MUTYH
APE1
DEC1
HIF1α
β-Tubulin
C
Protein (relative/β-tubulin)
Thigh
FLS IIIA
D
NHL IIB (n=8)
FLS IIIA (n=3)
Fraction
Positive cells, %
Count
CD45+ 44 +/- 8.1
CD20+ 34 +/- 5.6
CD34+ 1.8 +/- 0.2
CD45+ 2.4 +/- 0.2
CD20+ 0.3 +/- 0.1
CD34+ 0 +/- 0.0

Supplemental information

Supplemental Figures and Tables

Table S1. List of oligonucleotide primers used in RT-qPCR (designed with Primer 3 software: http://frodo.wi.mit.edu/primer3/)

Symbol	Accession#	Primers (5'->3') (Start-End)	$P_{L,\ bps}$
Core BER genes			
POLL	NM_001174084.1	FW: cctgtgccctgctctacttc RV: ctcagcaggttctcggtagg (1994-2211)	218
POLB	NM_002690.1	FW: gagaagaacgtgagccaagc RV: cgtatcatcctgccgaatct (189-392)	204
hOGG1	NM_002542.5	FW: gcagcagctacgagagtcct RV: ttcccagttccttgttggtc (1018-1244)	227
MUTYH	NM_001048171.1	FW: ccagagagtggagcaggaac RV: tttctggggaagttgaccac (989-1144)	156
APE1	NM_001244249.1	FW: gctgcctggactctctcatc RV: gctgttaccagcacaaacga (662-841)	180
Hypoxia markers			
HIF1α	NM_002690.1	FW: gaaagcgcaagtcctcaaag RV: tgggtaggagatggagatgc (2175-2341)	167
HIF2α	NM_001430.4	FW: ttgatgtggaaacggatgaa RV: ggaacctgctcttgctgttc (2713-2908)	196
CAIX	NM_001216.2	FW: taagcagctccacaccctct RV: tctcatctgcacaaggaacg (1101-1350)	250
DEC1	NM_017418.2	FW: ccttgaagcatgtgaaagca RV: gcttggccagatactgaagc (606-785)	180
GLUT1	NM_006516.2	FW: cttcactgtcgtgtcgctgt RV: tgaagagttcagccacgatg (1482-1711)	230
Angiogenesis markers			
VEGF	NM_001025366.2	FW: cccactgaggagtccaacat RV: tttcttgcgctttcgttttt (1324-1509)	186
bFGF	NM_002006.4	FW: ggtgaaaccccgtctctaca RV: tctgttgcctaggctggact (2939-3110)	172
TGF-β	NM_000660.4	FW: gggactatccacctgcaaga RV: cctccttggcgtagtagtc (966-1204)	239
Reference genes			
RPL28	NM_000991.4	FW: gcaattccttccgctacaac RV: tgttcttgcggatcatgtgt (146-343)	198

Table S2. List of primary antibodies

Symbol	Entrez-Gene ID/Protein	Mw, kDa	Cat. #	Manufacturer
Core BER genes				
POLL	27343, polymerase (DNA directed), lambda	72		Locally raised
POLB	5423, polymerase (DNA directed), beta	38	PA1-23198	Thermo Scientific
hOGG1	4968, 8-oxoguanine DNA glycosylase	39	NB100-106	Novus
MUTYH	4595, mutY homolog (E. coli)	65	sc-30630	Santa Cruz
APE1	328, APEX nuclease (multifunctional DNA repair enzyme) 1	37	sc-5572	Santa Cruz
Hypoxia markers				
HIF1α	3091, hypoxia inducible factor 1, alpha subunit	120	610958	BD
CAIX	768, carbonic anhydrase IX	58	PA1-16592	Thermo Scientific
DEC1	50514, deleted in esophageal cancer 1	51	A300-649A	Betyl
GLUT1	6513, solute carrier family 2, member 1	55	NB110-39113	Novus
Others				
Catalase	847, catalase	59	ab1877	Abcam
GSH-Px	2876, glutathione peroxidase 1	22	ab22604	Abcam
β-tubulin	203068, tubulin, beta class 1	55	T8328	Sigma
$P70S6K_{Thr389}$	6198, RP S6 kinase	70	9205	Cell Signaling
8-oxo-G			MAB3560	Millipore

Supplemental Experimental Procedures

In silico identification of hypoxia responsive elements
Identification of hypoxia responsive elements (HRE) in promoters of base excision repair genes was performed as described in (Qiu et al., 2003) with modifications. Gene IDs along with sequence information were retrieved from NCBI Gene (http://www.ncbi.nlm.nih.gov/gene/) and the eukaryotic promoter database (EPD, http://epd.vital-it.ch/) was used to extract promoter sequences (identified promoters), or alternatively, 10 kbps 5'- upstream sequences were obtained from Genatlas (http://genatlas.medecine.univ-paris5.fr/) and processed by BDGB (http://www.fruitfly.org/) and Pro Scan (http://bimas.dcrt.nih.gov/molbio/) network for promoter prediction (unknown promoters). Signal Scan (http://bimas.dcrt.nih.gov/molbio/) analyzed extracted and predicted promoter sequences.

Reporter gene assay
The assay for reporter gene was performed as described in (Wollenick et al., 2011) with minor modifications. Plasmid vectors provided by these authors were digested with Mlu1 and Xho1 and ligated to either wild-type POLB (5' – cgcgtCCGCCCCGCCCAGGACGCGTGACGTCACAACAACGCc – 3', 3' – tcgagGCGTTGTTGTGACGTCACGCGTCCTGGGCGGGGCGGa – 5') and hOGG1 (5' – cgcgtAACGGGCTGGGGAAGGACGTGGCTCTGAAG
ACGGACc – 3', 3' – tcgagGTCCGTCTTCAGAGCCACGTCCTTCCCCA
GCCCGTTa – 5') or mutant POLB (5' – cgcgtCCGCCCCGCCCAGGACtat
caACGTCACAACAACGCc – 3', 3' – tcgagGCGTTGTTGTGACGTtgataG
TCCTGGGCGGGGCGGa – 5') and hOGG1 (5' – cgcgtAACGGGCTGGG
GAAGGtatcaGCTCTGAAGACGGACc – 3', 3' – tcgagGTCCGTCTTCAG
AGCtgataCCTTCCCCAGCCCGTTa – 5') synthetic oligonucleotides, representing respective gene promoters with intact (WT) or disrupted (mut) HRE. Transfections for reporter gene experiments were carried out on 100 mm culture plates, 293 cells were co-transfected with 3 µg reporter plasmid or a mix of 1.5 µg reporter and 1.5 µg expression plasmids, respectively. The transfection efficiency was controlled by co-transfection of 30 ng pEGFP-C2 (Clontech).

Isolation and culture of cells from dog tumors and tissues
Tumor and non-transformed cells were isolated from excised tissues of dogs undergoing surgery as described in (Quero et al., 2011) with modifications. Briefly, tissue samples were washed twice with ice cold PBS and homogenized in the sterile 0.3% collagenase NB4 (Cat. #17454, Serva, Switzerland) and 0.2% dispase II (Cat. #04942078001, Roche Diagnostics, Switzerland) in PBS for several hours. The cells were filtered through a 70 µm cell strainer (BD Bioscience, Switzerland) and expanded in monolayer culture system containing DMEM/F12 (Sigma, Switzerland) media with 10% FCS (Cat. #S0400-500, BioWest, Switzerland), penicillin (50 U/ml), streptomycin (50 µg/ml), and ampicillin (125 ng/ml) (Invitrogen, Switzerland). Medium was changed twice a week. Cells were used for experiments in passage 2 or 3.

MTT assay

Cell viability was determined by the MTT assay according to (Mosmann, 1983) with some modifications. Briefly, cells were seeded in 96-well plate at a density of 10^4 cells per well and let to adhere overnight. Next, the cells were subjected to different oxygen conditions and subsequently treated as described throughout the text and Figure legends. Untreated cells served as controls. After treatment, the cells were washed twice with PBS solution and incubated in DMEM (180 µl) containing 500 µg/ml 3- [4,5-dimetho-thiazol-2-yo]-2,5-diphenyl tetrazolium bromide (MTT) (Fluka, St. Louis MO, USA) for 4 h at 37 °C. The medium containing MTT was then removed and replaced with 150 µl dimethyl sulphoxide (DMSO). The plate was agitated for 2 min allowing MTT-formazan to dissolve into DMSO completely. The absorbance was determined at 570 nm using a Tecan C200 Micro plate Reader. Cell viability was calculated according to the equation: Cell viability (%) = (the absorbance of experiment group/the absorbance of control group) ×100%. Cell viability at each tested condition was obtained from 8 parallel wells. The experiment was repeated three times.

Measuring catalase activity

The catalase activity of cell lysates was determined according to (Li and Schellhorn, 2007) with modifications. Decrease in H_2O_2 level was monitored at 240 nm in a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2 with 20 µg of total cell lysate (WCE). Catalase from bovine liver (Cat. #C30-100MG, Sigma-Aldrich, USA) was used as a standard. The specific inhibitor of catalase 3-AT was used to ensure the specificity of the assay (Darr and Fridovich, 1986). 5 mM 3-AT inhibited the catalase activity over 90 %.

Measuring glutathione peroxidase (GSH-Px) activity

The GSH-Px activity was measured according to (Flohe and Gunzler, 1984) in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 1 mM GSH, 10 mM H_2O_2, 2.5 U/ml GSH reductase, 150 pM NADPH, and 20 µg of whole cell protein extract. Consumption of NADPH was monitored at 340 nm. The GSH-Px activity was inhibited by 1.8 mM N-ethyl-maleimide (NEM) (Gregory et al., 1971). To ensure that NEM did not affect GSH reductase, oxidized GSH was added to the reaction mixture after activity determination.

Supplemental References

Darr, D., and Fridovich, I. (1986). Irreversible inactivation of catalase by 3-amino-1,2,4-triazole. Biochem Pharmacol *35*, 3642.

Flohe, L., and Gunzler, W.A. (1984). Assays of glutathione peroxidase. Methods Enzymol *105*, 114-121.

Gregory, E.M., Yost, F.J., Jr., Rohrbach, M.S., and Harrison, J.H. (1971). Selective chemical modification of malate dehydrogenase. N-ethylmaleimide modification of active center sulfhydryl residues. J Biol Chem *246*, 5491-5497.

Li, Y., and Schellhorn, H.E. (2007). Rapid kinetic microassay for catalase activity. J Biomol Tech *18*, 185-187.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65*, 55-63.

Qiu, P., Qin, L., Sorrentino, R.P., Greene, J.R., Wang, L., and Partridge, N.C. (2003). Comparative promoter analysis and its application in analysis of PTH-regulated gene expression. J Mol Biol *326*, 1327-1336.
Quero, L., Klawitter, M., Nerlich, A.G., Leonardi, M., Boos, N., and Wuertz, K. (2011). Bupivacaine--the deadly friend of intervertebral disc cells? The spine journal : official journal of the North American Spine Society *11*, 46-53.
Wollenick, K., Hu, J., Kristiansen, G., Schraml, P., Rehrauer, H., Berchner-Pfannschmidt, U., Fandrey, J., Wenger, R.H., and Stiehl, D.P. (2011). Synthetic transactivation screening reveals ETV4 as broad coactivator of hypoxia-inducible factor signaling. Nucleic Acids Res.

IV. UNPUBLISHED RESULTS

IV.1. THE BER CAPACITY AND CELL SURVIVAL UPON HYPOXIA ARE REGULATED VIA AKT DEPENDENT PHOSPHORYLATION OF hOGG1

IV.1.1. BACKGROUND

The expression of BER genes has been shown to be regulated in a complex manner often involving the integration of multiple pathways and levels of regulation (Zharkov, 2008). Previously, it has been demonstrated that hypoxia, or precisely hypoxic preconditioning, activated expression of hOGG1 and Pol β in a HIF dependent manner (Razumenko M., 2012) (see Manuscript on page 24.). Remarkably, both mRNA and protein levels of hOGG1 and Pol β were elevated during 16 hours of hypoxia treatment and, in case of Pol β, reverted gradually to its basal values, during 120 min of re-oxygenation. At the same time, hOGG1 protein levels were not significantly changed by re-oxygenation (III.1. Fig. 1, A-C). Further investigation of hOGG1 protein levels by immunoblotting, during hypoxia and re-oxygenation in different cell lines, identified a post-translation modification of hOGG1, which by band size and electrophoretic mobility shift in an SDS-PAGE could be classified, accordingly to literature, as phosphorylation (Kovacs et al., 2003). Moreover, phosphorylation of hOGG1 protein was the only modification induced by hypoxia and was detected throughout 120 minutes of re-oxygenation, but, on the other hand, not at the start of hypoxic preconditioning. Knock-down of the hOGG1 gene using specific siRNA resulted in reduced mRNA and protein levels of hOGG1 gene, with proportional reduction of phosphorylation. It was, however, not completely eliminated, suggesting that HIF dependent elevation of hOGG1 expression, and its modification, are simultaneous, and possibly, independent events, since HEK293 cells that were stably transfected with shRNA for HIF-1α and were unable to induce hOGG1expression under hypoxia, were still able to phosphorylate the hOGG1 protein.

In the literature, multiple modification of hOGG1 protein has been reported, although, not in the context of hypoxia, or hypoxic preconditioning (Hill et al., 2008) (Bhakat et al., 2006). In spite that phosphorylation of hOGG1 has been described as the most frequent modification, only few reports analyzed its influence on the regulation and function of hOGG1 (Hill et al., 2008). However, neither exact motives, nor pathways responsible for hOGG1 phosphorylation have been identified in the literature so far. Consequently, the biological role of the specific hOGG1 phosphorylation has been explored so far only to a limited extent.

IV.1.2. MATERIALS AND METHODS (IN ADDTION TO III.1)

IV.1.2.1. CHEMICALS AND ANTIBODIES

Isoform selective inhibitor of Akt VIII (Cat. #124018, Merck, Darmstadt, Germany) and inhibitor of PI3K GDC-0941 (Cat. #S1065, LuBioScience, Lucerne, Switzerland) were of analytical grade. Oligonucleotides for site-directed mutagenesis of hOGG1 (**Table 2**) and for RT-qPCR (**Table 3**) were synthesized by Mycrosynth (Balgach, Switzerland) and antibodies (**Table 4**) were purchased from different suppliers.

Table 2: List of oligonucleotides used for site-directed mutagenesis of hOGG1

Code	Mutant	Oligonucleotides (5'->3') (Start-End)	
Human OGG1, NM_002542.5			
S44A	Ser44 -> Ala44	FW: CCTTCTGGACAAGCTTTCCGGTGGAG RV: CTCCACCGGAAAGCTTGTCCAGAAGG	
S51A	Ser51 -> Ala51	FW: GTGGAGGGAGCAAGCTCCTGCACACTG RV: CAGTGTGCAGGAGCTTGCTCCCTCCAC	
S115A	Ser115 -> Ala115	FW: GTATCACCACTGGGGTGCCGTGGACTCCCACTTC RV: GAAGTGGGAGTCCACGGCACCCCAGTGGTGATAC	
S211A	Ser211 -> Ala211	FW: GTTACGTGAGTGCCGCTGCCCGAGCCATC RV: GATGGCTCGGGCAGCGGCACTCACGTAAC	
S340A	Ser340 -> Ala340	FW: CGCAGAAAGGGTGCCAAAGGGCCGG RV: CCGGCCCTTTGGCACCCTTTCTGCG	

Table 3: List of oligonucleotide primers used in RT-qPCR

Symbol	Accession#	Primers (5'->3') (Start-End)	$P_{L,\ bps}$
PKB (Akt) genes			
Akt1	NM_001014431.1	FW: catcacaccacctgaccaag RV: ctcaaatgcacccgagaaat (1684-1884)	201
Akt2	NM_001243027.1	FW: gaggtcatggagcacaggtt RV: ctggccgagtaggagaactg (1466-1695)	230
Akt3	NM_001206729.1	FW: cagtagactggtggggccta RV: atcaagagccctgaaagcaa (1130-1298)	169

Table 4: List of primary antibodies

Symbol	Entrez-Gene ID/Protein	$Mw_{,\ kDa}$	Cat. #	Manufacturer
PKB (Akt)				
Akt1	207, v-akt murine thymoma viral oncogene homolog 1	60	2938	Cell Signaling
Akt2	208, v-akt murine thymoma viral oncogene homolog 2	60	5239	Cell Signaling
Akt3	10000, v-akt murine thymoma viral oncogene homolog 3	60	4059	Cell Signaling
$PAkt_{Ser473}$	Activated form of Akt (isoform unspecific antibody)	60	9271	Cell Signaling
Others				
Phosphoserine	phosphorylated serine amino acid		P5747	Sigma
HA-tag	human influenza hemagglutinin (HA)		05-904	Millipore

IV.1.2.2. SITE DIRECTED MUTAGENESIS, CELL FRACTIONATION, AND IMMUNOPRECIPTATION

Site-directed mutagenesis and preparation of whole protein extracts for cell fractionation experiments were performed as described by Markkanen et al. (Markkanen et al., 2012). Immunoprecipitation of hOGG1 was performed using monoclonal anti-HA agarose conjugate (Clone HA-7) (Cat. #2095, Sigma, Bülach, Switzerland) accordingly to manufacturer's instructions.

IV.1.3. RESULTS

IV.1.3.1. PHOSPHORYLATION OF hOGG1 UPON HYPOXIA IS AKT DEPENDENT

So far phosphorylation of hOGG1 has been identified on Ser340, protein kinase C (PKC) can carry out this (Dantzer et al., 2002). Thus, the primary question in this project was to identify putative phosphorylation sites of, along with potential kinase(s).

By using NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) *in silico* prediction with mid-stringency thresholds ($\nu \geq 0.55$) five putative phosphorylation motives for 3 respective kinases were identified in the amino-acid sequence of hOGG1 (Fig. 15, A). The modified amino acid in all motives was serine, located in positions 44, 51, 115, 211 and 340, respectively. S44, S115 and S211 were putative sites for protein kinase C (PKC), S51 for PKB and S340 for PKA, respectively. All kinases represent, however one family – ABC kinases (Newton, 2003). Remarkably, the only serine in position 51 formed so called SP-site known for its ability to distort secondary and tertiary protein structures upon phosphorylation, and thus to be detected as a corresponding band shift in SDS-PAGE (Kovacs et al., 2003). The role of each potential phosphorylation site was further investigated *ex vivo*. For this reason, all five serine residues were mutated to alanine by site-directed mutagenesis (Fig. 15, B). The resulted plasmid constructs were transfected into HEK293 cells 48 hours prior to hypoxic preconditioning. Only the S51A mutant was detected with reduced phosphorylation after 16 hours of hypoxia, as compared with its wild type (WT) (Fig. 15, C), indicating an involvement of PKB (Akt) in phosphorylation of hOGG1. In the next step, specific siRNA were used to knock-down the expression of the PKB (Akt) kinase. Since the PKB family includes three members, being Akt1, Akt2, and Akt3, representing, 85%, 10% and 5 %, respectively, of PKB pool in the cell (Bellacosa et al., 2004), three different siRNA were used (Qiagen IDs: s102757244, s100287742, s101140326) (Fig. 15, D-E). Phosphorylation of WT hOGG1 under hypoxia was not affected by down- regulation of Akt1. On the other hand, down regulation of Akt2 resulted in reduced hOGG1 phosphorylation. The Akt3 mRNA could not be detected in HEK293 cells (Fig. 15, E-F). In order to determine, whether activation of Akt, is required to phosphorylate hOGG1, the selective Akt kinase family inhibitor VIII, referred as inhibitor 8 (INH8), was used to block activation of PKB *ex vivo* in a dose dependent manner. Treatment of HEK293 hOGG1WT cells with 2 μM of inhibitor 8 during the last 2 of 16 hours of hypoxia, prevented activation of all three Akt 1, 2 and 3 isoforms. This resulted in a 10 fold reduced hOGG1 phosphorylation. On the other hand, no change in hOGG1 phosphorylation was detected when INH8 was used at 200 nM or lower. This dose has been reported in the literature to inhibit the activation of the predominant Akt1 isoform, but without affecting other two, Akt2 and Akt3 (Zou et al., 2009) (Fig. 15, F), suggesting that phosphorylation of hOGG1 might be induced by Akt2.

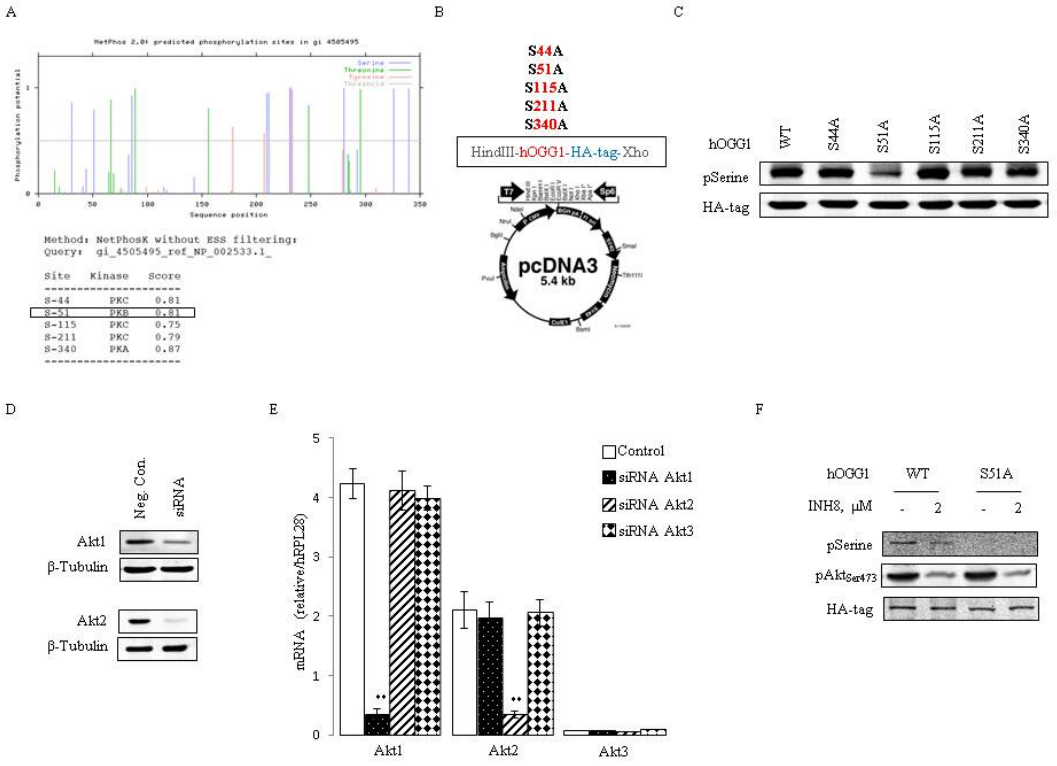


Fig 15: Phosphorylation of hOGG1 under hypoxia is PKB (Akt) dependent. (A) *In silico* prediction of putative phosphorylation sites in the hOGG1 protein. Formatted output of NetPhosK 1.0 algorithm shown in the lower part, and SP-site is marked by black square (refer to the text for details); (B) SDM generated hOGG1 constructs; (C) Phosphorylation of hOGG1 mutant constructs under hypoxia (0.2% O_2) analyzed by immunoblotting. Representative experiment is shown; (D) Silencing of PKB (Akt) genes under hypoxia (0.2% O_2). HEK293 cells were transfected with respective siRNA 48 hours prior to the treatment; (E) Expression of PKB (Akt) mRNAs in HEK293 cells under hypoxia (0.2% O_2) analyzed by RT-qPCR; (F) Phosphorylation of hOGG1 mutant constructs under hypoxia (0.2% O_2) and treatment with INH8 analyzed by immunoblotting. Representative experiment is shown;

IV.1.3.2. PHOSPHORYLATION OF hOGG1 MODULATES ITS ACTIVITY AND CELLULAR LOCALIZATION DURING HYPOXIA

Since it has been reported that phosphorylation of hOGG1 might alter its activity and localization in the cell (Dantzer et al., 2002), the functional role of hypoxia induced hOGG1 phosphorylation was next tested. For this the cellular localization of hOGG1 during hypoxic preconditioning was analyzed by immunofluorescence. Over 73.2+/-4.9% ($p<0.05$) of the hOGG1 protein was translocated from the cytoplasm to the nucleus upon 16 hours of hypoxia, and a significant portion of that (51+/-6.4%, $p< 0.05$) remained within the nuclear compartment following 120 min of re-oxygenation (Fig. 16, A-B). Independently, analysis of cell fractionations after transfection of HEK293 cells with WT or mutated hOGG1 constructs indicated that the S51A mutant, that cannot be phosphorylated, was not present in nuclear fraction, and remained entirely within cytoplasm fraction, either during 16 hours of hypoxia, or 120 min of re-oxygenation, suggesting phosphorylation might be important for hOGG1 translocation (Fig. 16, C). The nuclear translocation of WT, but not S51A mutant, was reduced by 90% when cells were treated with 2 μM of INH8 during hypoxia, suggesting that Akt affects by phosphorylation of hOGG1 its cellular localization. When HEK293 whole cell extracts (WCE), that were transfected with WT and mutant hOGG1 plasmid constructs, were compared in an *in vitro* BER assay, WCE of WT hOGG1 protein after hypoxic precondition had an 8 fold higher BER activity than either the S51A mutant under

hypoxia, or the WT hOGG1 protein without hypoxic preconditioning (Fig. 16, D). Again, treatment of HEK293 WT hOGG1 cells, during hypoxic preconditioning, with 2 μM of INH8, returned the activity of hOGG1 mediated BER to the levels observed for S51A mutant under hypoxia, or for the WT hOGG1 without hypoxic preconditioning (Fig. 16, E). Moreover, knock-down of Akt2, but not Akt1 with specific siRNA reduced the activity of hOGG1 WT mediated BER by 7 fold (Fig. 16, F-G), suggesting that the BER capacity is indeed dependent on Akt2.

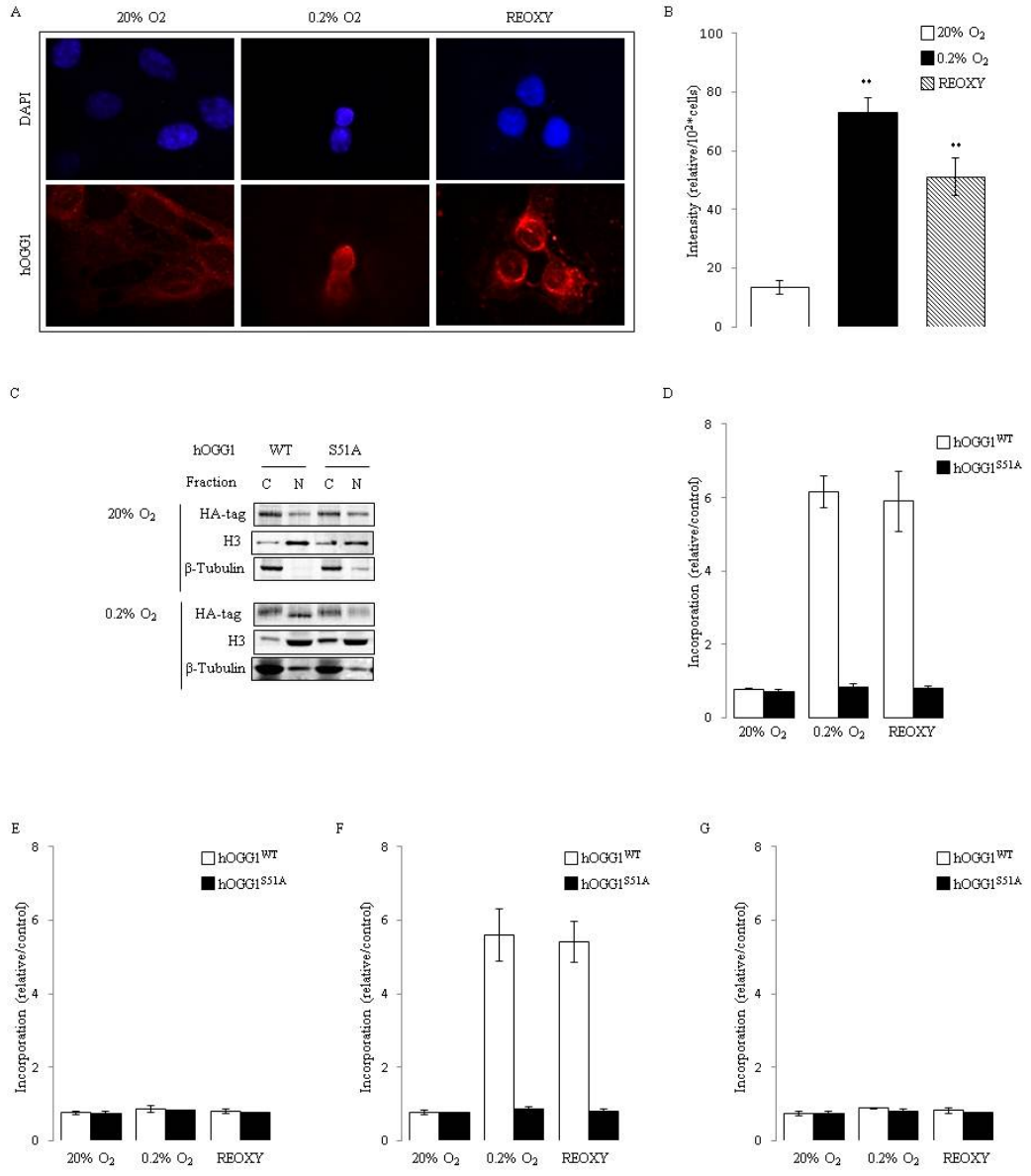
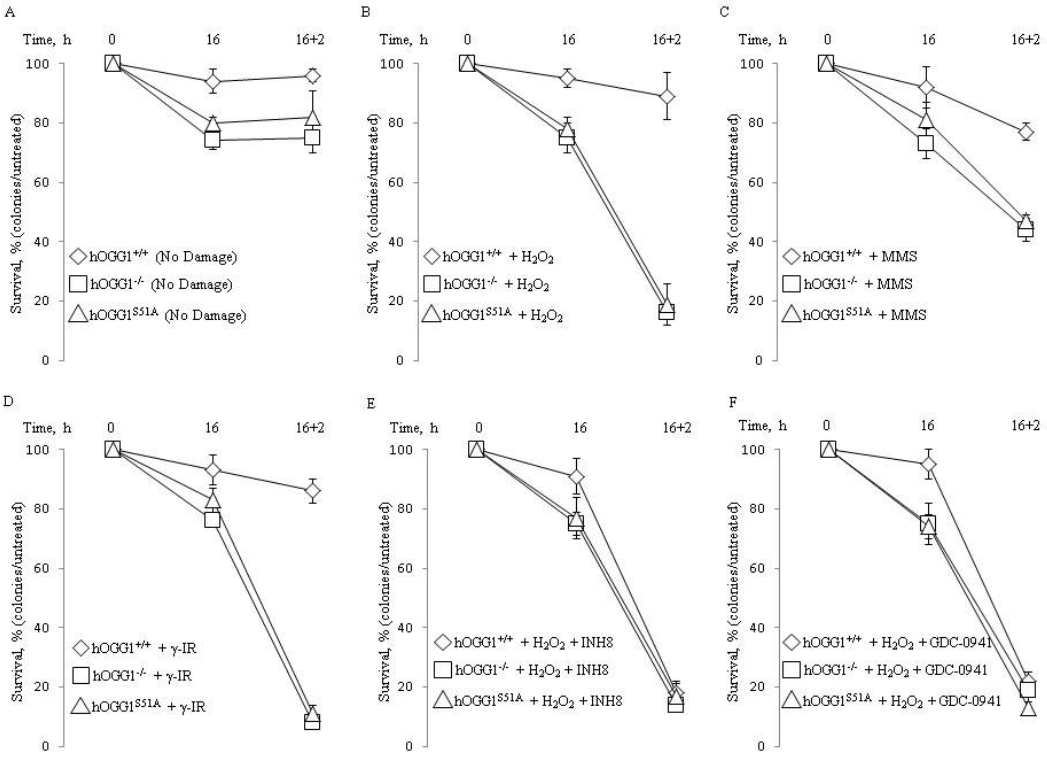


Fig. 16: Phosphorylation of hOGG1 modulates its activity and cellular localization during hypoxia. (A) Intracellular localization hOGG1 analyzed by immunofluorescence with a specific antibody. (B) Mean fluorescence intensity of nuclear hOGG1 fraction calculated for 200 cells +/- SEMs in A. (C) Intracellular localization hOGG1 upon hypoxia analyzed by cell fractionation and immunoblot. Representative samples are shown. (D) *In vitro* BER assay of HEK293 WCEs transfected with WT and hOGG1 mutants and cultured for 16 hours at respective oxygen atmosphere. (E) Same as in F but cells were treated with INH8, or (F-G) PKB (Akt) was silenced by specific siRNA. Shown are mean values of incorporation of radioactively labeled [α-P^{32}] dGTP on S2 subtracted to control +/- SEMs of three experiments.

IV.1.3.3. PHOSPHORYLATION OF hOGG1 IN RESPONSE TO HYPOXIA IS NECESSARY TO PROMOTE CELL SURVIVAL AND RESISTANCE TO DNA DAMAGING AGENTS

In order to investigate role of hOGG1 phosphorylation in cell survival a colony forming assay was used. Initially, the survival of mouse embryonic fibroblasts (MEF) knocked-out for hOGG1 gene (MEF $OGG1^{-/-}$) was compared to the survival of MEFs complemented by the WT hOGG1 plasmid, or by the hOGG1 phosphorylation mutants during hypoxia and re-oxygenation. Significantly lower (29.8+/-2.8%, $p< 0.05$; 24.1+/-1.3%, $p<0.05$) number of colonies were counted past 7 days after hypoxic precondition, for both, $hOGG1^{-/-}$ cells or the same cells transfected with S51A mutant, as compared to the MEFs complemented with WT hOGG1 ($hOGG1^{+/+}$) construct (Fig. 17, A). Moreover, treatment of $hOGG1^{-/-}$ cells or $hOGG1^{-/-}$ cells transfected with the S51A construct, at either 1 mM H_2O_2 (Fig. 17, B), 100 mM of methyl methanesulfonate (MMS) (Fig. 17, C) or 3 Gy of γ-irradiation (Fig. 17, D) during 120 minutes of re-oxygenation, resulted in further decrease of colony counts. $hOGG1^{+/+}$ cells were tolerating such a treatment at a large extent and only a negligible reduction in colony counts was observed (Fig. 17, B-D). This suggested that phosphorylation of hOGG1 is required for survival. Furthermore, treatment of $hOGG1^{+/+}$ cells with 2 μM of INH8 (Fig. 17, E-G), or with 100 nM of GDC-0941 (Fig. 17, H-J), a specific inhibitor of PI3K kinase acting upstream of PKB during hypoxia, reduced survival after treatment with damaging agents to levels comparable with survival of $hOGG1^{-/-}$ MEFs or the $hOGG1^{-/-}$ MEFs transfected with the S51A mutant, suggesting survival and stress tolerance are dependent on the active PKB signaling. Decreased survival in response to treatment with DNA damaging agents was also observed when $hOGG1^{+/+}$ cells were transfected with siRNA for Akt2, but not with siRNA for Akt1 (Fig. 17, K-P)



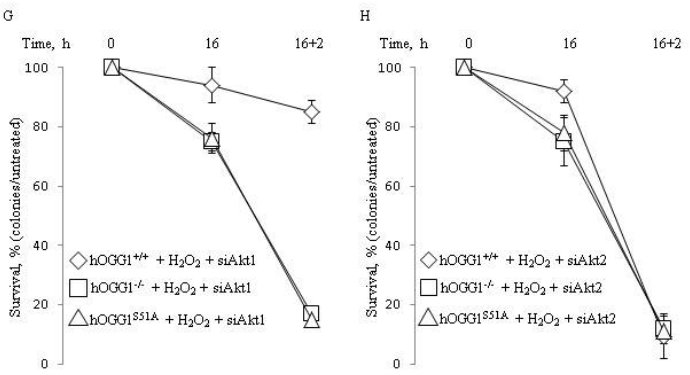


Fig. 17: Phosphorylation of hOGG1 in response to hypoxia is necessary to promote cell survival and resistance to DNA damaging agents. Colony formation assay of MEF hOGG1$^{+/+}$, hOGG1$^{-/-}$ and hOGG1^{S51A} cells (A) subjected to hypoxia and re-oxygenation, or treated with (B) 1 mM H_2O_2 (C) 100 nM MMS (D) 3 Gy of γ-irradiation, during 120 minutes of re-oxygenation; (E-G) threated the same as in B-D plus INH8 (H-J) threated the same as in B-D plus GDC-0941; (K-P) threated the same as in B-D plus PKB (Akt) siRNA mediated silencing. The mean values +/- SEMs of three experiments are shown.

IV.1.3.4. EXPRESSION OF hOGG1 AND AKT ISOFORMS IS ELEVATED IN HUMAN TUMORS

As the next step, expression of hOGG1 and PKB in human tumors was analyzed. Splenic marginal zone lymphoma (SMZL) was selected for this reason, as a variant of human tumor, that is, by histology and pathogenesis, closely related to non-Hodgkin, non-metastatic lymphomas, derived from spleen of dogs and previously analyzed in this thesis (Section III.1., Fig. 4, A-C). Grade N2 of SMZL was considered as comparable, by morphological changes, to the grade IIB, of mentioned dog's NHL. Tumor biopsies, in the case of SMZL, were also matched to non-transformed splenic tissue, derived during surgery. Five matched pairs were used to analyze mRNA levels of hOGG1, Pol β and PKB (Akt), isoforms 1 (Akt1) and 2 (Akt2) (Fig. 18, A). Levels of mRNA for all four studied genes were elevated 6.12, 3.26, 2.73 and 4.94 fold, respectively, in SMZL samples as compared to non-transformed tissue (Fig. 18, A). The values were normalized in all cases to ribosomal protein L28 (RPL28) and also to the content of $CD20^+$ B-cells in non-transformed (Fig. 18, B, left) and tumor (Fig. 18, B, right) tissues. Simultaneously, increased expressions of both hOGG1 and the two PKB isoforms might be in this case an indirect indication for their dependent involvement in tumor genesis. This conclusion, however, urges for further investigation.

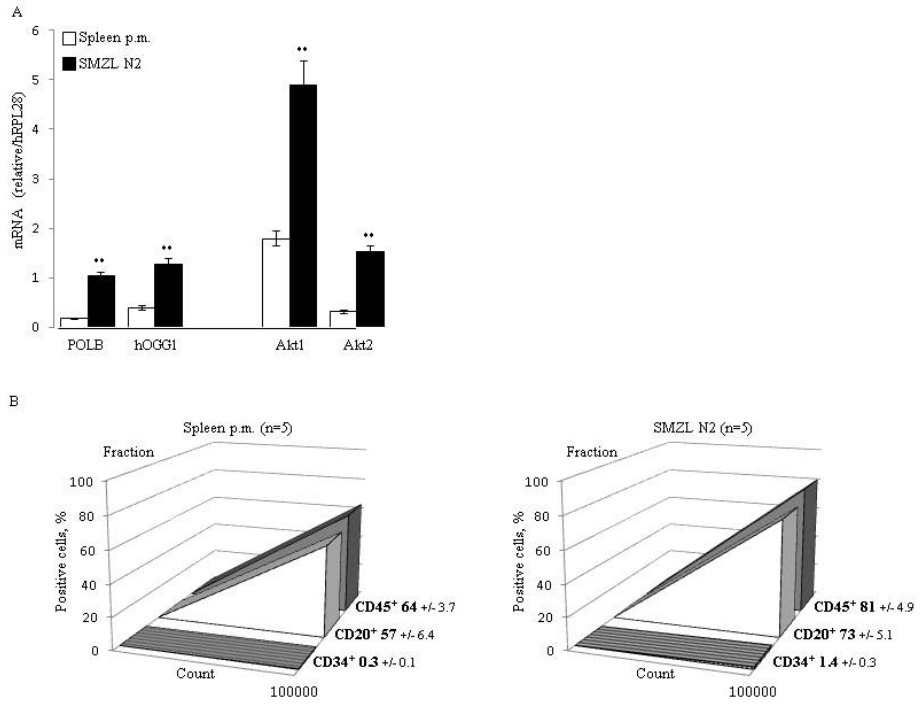


Fig. 18: Expression of hOGG1 and Akt isoforms is elevated in human tumors (A) mRNA expression levels of hOGG1, POLB and two PKB (Akt) genes in SMZL samples (n=5) analyzed by RT-qPCR. Shown are mean values +/- SEMs. (B) Fractionation of splenic post-mortem samples (left) and SMZL tumor samples (right) by flow cytometry. Percentage of positive cells is indicated, +/- SEM.

IV.1.4. CONCLUDING REMARKS AND PROSPECTIVES

Phosphorylation of hOGG1 has been described as the most frequent post-translational modification (Hill et al., 2008) introduced in response to multiple stimuli, including genotoxic stress (Bhakat et al., 2006). Frequent phosphorylation of the protein can partially be explained by its amino-acid composition. Thus, hOGG1 contains polar amino-acids in its primary structure in relatively high density, 11%. Among them are seventeen serine, five tyrosine and three threonine residues that can be phosphorylated (Ishida et al., 1999). This makes mapping and detailed characterization of the phosphorylation sites to be very complex, which is illustrated by the fact that only phosphorylation of Ser340 has been characterized and reported up to date (Dantzer et al., 2002).

In silico approaches are used to study phosphorylation, although subsequent verification of such results is required. Using NetPhosK algorithms, it was possible to identify five potential phosphorylation sites, one of which, Ser51, was phosphorylated by PKB (Akt) kinase in response to hypoxia, which also was confirmed by the analysis of non-phosphorylated hOGG1 mutants. Despite many advantages the NetPhosK service is limited to the use of pre-defined parameters set by the system, such as stringency value (ν) and sensitivity (ρ), misuse of which can generate false positive and/or false negative hits. In this context, whole proteome identification of phosphorylation sites by mass-spectrometry (MS) (Munton et al., 2007) is an alternative approach that allows identification

of phosphorylation sites *in vivo*. MS was also applied in this thesis project. These data have, however, not yet been available for this discussion.

In order to verify role of PKB in the phosphorylation of hOGG1, a loss-of-function experiment was performed. Using siRNA silencing of Akt kinase isoforms, Akt2 was identified to catalyze Ser51 phosphorylation in response to hypoxia. It was confirmed also by use of selective isoform specific inhibitor of PKB (Akt) and hOGG1 mutant constructs. However, *in vitro* phosphorylation of purified hOGG1 protein by activated PKB, isoform 1 (Akt1), have not resulted in any detectible hOGG1 phosphorylation, in contrast to the known substrate of PKB, p21 that was used as a positive control. Remarkably, the catalytic domain of PKB is highly conserved between all three isoforms, and main differences between isoforms consist of the regulatory C-terminal part of the protein (Bellacosa et al., 2004). In this context, failure to phosphorylate hOGG1 *in vitro*, on the one hand, might indicate inability to fully reconstitute parameters of the kinase reaction held *in vivo*, but on the other hand, it might also indicate an important role of protein-protein interactions in phosphorylation of hOGG1 under hypoxia. In prospective, generation of enzymatically inactive and/or truncated PKB (Akt) mutant constructs will be required to elucidate details of the mechanism of hOGG1 phosphorylation, which still remains only merely investigated.

V. SUMMARY OF THE MAJOR FINDINGS

V.1. Hypoxia activates expression of core BER genes.

- Pol λ (POLL), Pol β (POLB) and hOGG1 are up-regulated by hypoxia, and their increased expression is maintained during the whole period of re-oxygenation, in spite of gradual decline
- MUTYH and Ape-1 are O_2 irresponsive and, in absence of other factors, are constantly expressed independently of O_2 availability

V.2. HIF and PKB pathways, but not the amount of ROS or base damages in the cell, regulate core BER genes in response to hypoxia and re-oxygenation.

- Expression of Pol β and hOGG1 is regulated via HIF-1α, and expression of pol λ is controlled by the mTOR kinase.
- PKB isoform 2 (Akt2) phosphorylates hOGG1 protein in response to hypoxia promoting its nuclear translocation and increasing its enzymatic activity.

V.3. Up-regulated expression of core BER genes results in elevated capacity of hOGG1 and MUTYH initiated DNA repair, thus leading to excessive stress tolerance and increased cell survival upon genotoxic stress.

V.4. Activation of core BER genes in human and animal solid tumors, correlates with degree of tumor hypoxia, aggressiveness of the neoplasms, and contributes to the therapeutic resistance of the tumors.

VI. FINAL DISCUSSION

Oxygen, which constitutes 20% of the earth's atmosphere, is also the most abundant element in the human body (65%) (Maltepe and Saugstad, 2009).

Such prevalence of oxygen in the human body is partially due to high content of oxygen atoms in bioorganic molecules, used as building blocks for cellular and tissue structures. However, substantial part of oxygen in human body is molecular gas (O_2) dissolved in human fluids and indispensable for aerobic organisms to produce energy by oxidative phosphorylation in the mitochondria. In this process the reduction of O_2 to water by the mitochondrial electron transport chain enables the conversion of adenosine diphosphate (ADP) into adenosine triphosphate, ATP. The consequence of this reaction is the formation of reactive oxygen species, which in spite of their physiological value, can also lead to toxic effects and diseases if accumulated in excessive amounts (Bertram and Hass, 2008).

ROS are constantly produced in the cellular metabolism and the majority of them are readily detoxified via cellular antioxidant defense systems, except cases, known as oxidative stress, when production of ROS exceeds cell's ability to eliminate reactive intermediates or to repair the resulting damage (Valko et al., 2007).

Oxidative stress is often associated with normal physiological functions of cells and organism, like active tissue proliferation, organ regeneration or intensive physical activity (Fruehauf and Meyskens, 2007), but still majority of conditions are pathological, including the most severe – tumor development (Schumacker, 2006).

The main issue of oxidative stress is damage to DNA that affects genomic stability, and thus plays a key role in tumor initiation and maintenance, which is demonstrated by high content of mutations, arising from oxidative lesions, in human tumors (Campbell et al., 2010). In unperturbed cells, base excision repair (Zharkov, 2008) is the essential pathway to effectively combat oxidative DNA damages and in particular, the most mutagenic of it, 8-oxo-guanine, detected in high amounts during oxidative stress (Klaunig and Kamendulis, 2004). Therefore, oxidative stress is a critical cellular condition that requires BER activity to be adjusted in accordance to it extent.

Almost 80% of intracellular ROS are generated through oxidative phosphorylation, which is driven by partial O_2 reduction in mitochondria, thus fluctuations in O_2 availability and consumption are the primary cause of oxidative stress under physiological conditions and also the main reason why all eukaryotic organisms maintain an oxygen homeostasis (Semenza, 2010). If adaptation to low O_2, mediated by HIF has been extensively studied (Semenza, 2007), mechanisms to protect and repair the DNA of a cell working at higher or very often fluctuating O_2 concentrations, are still poorly understood (van Loon et al., 2010).

The aim for this thesis was to elucidate potential mechanisms that control expression of core BER genes in response to differential O_2 availability. In contrast to previously reported inactivation of DNA repair systems, including homologous recombination and non-homologous and joining (NHEJ) (Chan et al., 2008), at low O_2 concentration, expression of core BER genes and resulted BER capacity were increased under hypoxia and remain unaffected long after re-oxygenation. This might indicate differential involvement of DNA repair machines, which starts from inactivation of energy consuming pathways and switching on the pathways, like BER, needed to combat additional oxidative damage caused by restored O_2 supply. This hypothesis was further supported by involvement of HIF-1α and mTOR, key regulators of gene expression under hypoxia, in activation of core BER genes. More importantly in this aspect, that activation of BER genes and increase in BER capacity were uncoupled from the amount of ROS produced or the content of oxidative DNA lesions, thus allowing BER to be directly controlled via O_2 sensing and signaling roots and not via DNA damage, as in this case it would prevent BER from activation under hypoxia, and consequently lead to intolerance to oxidative damage raised either from re-oxygenation or oxidative

stress. Besides, the involvements HIF and mTOR, the BER capacity was also affected by posttranslational modifications. In particular, phosphorylation of hOGG1 via Akt2 increased activity and nuclear translocation of the protein, resulting in even higher BER capacity after re-oxygenation. This, on the one hand, might be just a second level in regulation BER function, but on the other hand, dual control mechanism that integrates also sensing of nutrients along to O_2 availability, could be general way of cell adaptation to chronic periods of hypoxia and re-oxygenation, which are common to many developmental processes.

Furthermore, activation of core BER genes by hypoxia that resulted in increase of the BER capacity was necessary to tolerate excessive DNA damage caused either by re-oxygenation or by re-oxygenation and genotoxic stress together. Using the siRNA approach hOGG1 and DNA pol β were identified as main factors of elevated DNA damage resistance. Remarkably the same mechanism of BER activation and the same BER genes were responsible for extreme resistance to DNA damaging agents, including therapeutic drugs, by re-oxygenated revived tumor cells *ex vivo*. Independently of the type or stage of tumor, and organism source, all solid tumors included in this thesis and their isolated cells were therapeutic resistant which correlated with their hypoxic status, expression of BER genes and of some pro-survival markers, like Akt2, highlighting the critical role of BER and hypoxia interplay in developing anti-cancer drug resistance. It was independently although indirectly supported by a recent report (Zhang et al., 2010).

The forces of BER activation *in vivo* are still awaiting further elucidation. In this direction, multiple reports highlight the importance of tissue O_2 dynamics. Impressively, the dynamics of perfusion and O2 persistence in the tissue ranges from just 20 milliseconds, in mouse brain under stress (Shen et al., 2009), to many days in a developing human embryo (Dunwoodie, 2009). Tumor tissue known for its chaotic vascularization is an excellent example. A high rate of cell proliferation in addition to dysfunctional perfusion constantly induces intermittent hypoxia in the growing tumor mass (Matsumoto et al., 2010), thereby activating BER genes. Finally, the littoral crab (*Carcinus maenas*), which can dive up to 40 meters deep and returns back to sea level, is repeatedly exposed to O2 concentrations between 0.01% and 20% for at least 30 times a day known for its high stress tolerance (Taylor, 1976). Conclusively, the mentioned processes would all be deleterious for living cells and organisms due to increased ROS production and high rate of oxidative DNA damage unless a general mechanism to combat that danger would exist as an “insurance” and can be adjusted accordingly to potential needs to sudden changes in the environment or in the body. Such changes in the body can be physiological as well as pathological (e.g. cancer microenvironment).

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Selected publications

1. Razumenko M, Stiehl, D.P., Wrann, S.G., Hofer-Inteeworn, N., Rohrer Bley, C., Wenger, R.H. and Hübscher, U. (2012) Hypoxia activates the base excision repair machinery promoting cell survival and tumor therapy resistance, submitted

2. Razumenko M. (2005) Phages Carrying Specific Peptides: Use in Diagnostics and Therapy, *Russian Patent Letters **12**: B-12*

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5. Razumenko M, Fedorova T, Lipkan D. (2003) Targeting of Peptides to Vascular Smooth Muscle Cells Under Different Pathological Conditions, *Archives of Clinical and Experimental Medicine **4**: 28-32*

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